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SOME POLYCLADS OF THE NEW ENGLAND COAST, ESPECIALLY OF THE WOODS HOLE REGION

LIBBIE H. HYMAN

(From the American Museum of Natural History, New York City)

This report is based on polyclads collected at Woods Hole, Massachusetts, Mt. Desert Island, Maine, Connecticut and Long Island, New York. I believe it includes practically all of the species expected to occur in the neighborhood of Woods Hole. The Woods Hole specimens were personally collected in the summer of 1935 or were loaned for identification by Mr. George Gray, Curator of the Museum of the Marine Biological Laboratory. The Peabody Museum of Yale University kindly loaned some specimens collected by Verrill along Massachusetts and Connecticut shores. The specimens from Mt. Desert Island, Maine, were personally collected in the summer of 1937. The specimens from Connecticut and Long Island were presented by Dr. Herman Spieth and Mr. M. D. Burkenroad.

Ten species are represented in the material; none of these is new but most of them are interesting as old species badly in need of reworking. Each species has been carefully studied whole and in serial section to determine its status according to modern taxonomic standards. It is gratifying to me to be able to clear up the taxonomic position of some of the old species of Girard and Verrill.

The scheme of classification here adopted is taken from Bock's invaluable treatise (1913) which is fundamental to all modern work on the group.

Before proceeding to the taxonomic descriptions, it seems desirable to give a brief account of the reproductive system of polyclads, since this system is of paramount importance in many genera. Polyclads are hermaphroditic with numerous testicular and ovarian follicles scattered throughout the parenchyma; there are no yolk glands. Fine vasa efferentia connect the testes to the paired vasa deferentia, expanded, thin-walled, usually sinuous canals, packed with sperm. These separately or after a union enter the male copulatory apparatus, which typically consists of seminal vesicle, prostate or granule vesicle, and penis. The seminal vesicle is a rounded, oval or curved sac with a thick muscular

wall. In some cases (*Stylochus* in part, some species of *Notoplana*) the terminal parts of the vasa deferentia acquire muscular walls and entirely resemble the seminal vesicle, so that a tripartite seminal vesicle results (Fig. 1). Certain glands known as granule (German workers) or prostate (English workers) glands are constantly associated with the male apparatus of polyclads. They may open directly into the terminal part of the male canal but more often are associated into a definite rounded or oval body with a muscular wall, termed the granule or prostate vesicle. The granule glands are often included inside this vesicle or may open into it from the exterior (extracapsular glands of Bock) or both. The granule vesicle may form a direct part of the male canal so that the sperm must pass through its lumen as in the Leptoplanidae (Fig. 5); this condition of the vesicle will here be termed "interpolated" ("eingeschaltet" in German). In some acotylean families and in the Cotylea in general the granule vesicle is "free," that is, connects by a duct to the male canal (Fig. 1). The function of the granule glands is unknown but the secretion is ejaculated with the sperm and must be of importance in their physiology. After passing through or receiving separately the granule vesicle, the male duct enters the penis proper, typically having the form of a projecting conical muscular papilla. It is frequently provided with a long or short hard stylet, sometimes curved or coiled, and may then itself be much reduced. The penis papilla may project directly into the male atrium or antrum or it may be at the inner end of a long canal leading from the atrium; this canal is termed by Bock the penis pocket (Fig. 5). When a penis pocket is present there may be at the place where the pocket widens into the male atrium, a penis-like projection, the penis sheath. In the family Planoceridae there is no proper penis but instead a cirrus, armed with teeth or spines. Penis here is limited to a male copulatory organ which is simply protruded by muscular action; cirrus means a copulatory organ which is everted when used so that the lining is turned to the outside.

The ripe eggs commonly collect in paired thin-walled expanded canals, the uteri, which in the family Leptoplanidae unite in front of the pharynx. The uteri, separately or after a union, open into the common unpaired female canal which then proceeds to the genital pore. This canal from the genital pore to the entrance of the uteri is commonly muscular and receives innumerable cement glands; Bock terms it vagina since it must serve for copulation in most polyclads. It is also the passage by which the eggs reach the exterior. Near the genital pore it may be widened into a cement pouch into which most or all of the cement glands open. The cement glands secrete the material by which the eggs are fastened into ribbons or masses and stuck to objects. In many poly-

clads the female canal continues inward proximal to the entrance of the uteri and terminates in a small or large spherical, oval, or tubular sac, now known as Lang's vesicle (Fig. 5). This appears to be a seminal receptacle since sperm are often seen in it.

The male apparatus is always anterior to the female apparatus. The genital pores are commonly separate but may be united. Paired or multiple male apparatuses are not uncommon.

Suborder Acotylea Lang 1884

Definition.—Polyclads without a sucker behind the female genital pore; pharynx ruffled; copulatory apparatuses behind the middle of the body; tentacles when present of the nuchal type; uteri anterior to the female genital pore.

Section Craspedommata Bock 1913

Definition.—Acotylea with marginal eyes, in addition to the usual cerebral and tentacular eyes.

Family Stylochidae Stimpson 1857 (emend. Bock, 1913)

Definition.—Craspedommata with a band of eyes along the whole or part of the body margin; female or both genital pores very near the posterior end; granule vesicle free; tentacles generally present; body usually oval, thick, firm and opaque.

Genus Stylochus Ehrenberg 1831 (emend. Lang 1884)

Definition.—Stylochidae with male and female pores close together in the posterior fourth of the body; tentacles well-developed; pharynx large with well-marked folds; prostate vesicle large, chambered, with extracapsular gland cells; seminal vesicle single or tripartite; Lang's vesicle absent.

Stylochus zebra (Verrill) 1882

Syn. *Stylochopsis zebra* Verrill 1882.
Stylochus zebra (Verrill) 1892.

Description.—The external features of this familiar Woods Hole polyclad have been adequately described by Verrill (1892). The body is of elongated oval or oblong form, obtuse at each end, of thick and firm texture, reaching a length of 30–40 mm. The dorsal surface has a color pattern of alternating yellowish or white and brown or chocolate cross-bars of which the most anterior and posterior ones are V-shaped. Near the anterior end are two rounded tentacles filled with eyes and between the tentacles are the paired cerebral groups of eyes. These

are loose elongated groups extending behind and before the level of the tentacles. The margin is completely encircled with a band of small eyes, wider in front, diminishing caudally.

Study of sections of the copulatory apparatus proves for the first time that this species was correctly placed by Verrill in the genus *Stylochus*. The uteri and vasa deferentia course alongside the posterior part of the very large, much ruffled pharynx behind the posterior end of which occurs the copulatory apparatus. The rear ends of the vasa deferentia expand and acquire muscular walls as they ascend to join the seminal vesicle proper, producing the three-parted type of seminal vesicle characteristic of several species of *Stylochus* (Fig. 1). The central lobe of this or seminal vesicle proper continues posteriorly as the ejaculatory duct which narrows and becomes less muscular, receiving near the penis the duct of the prostate vesicle. The latter is a large oval chambered sac with thick muscular walls penetrated by the necks of extracapsular granule glands (Fig. 1). The erect position of the prostate vesicle is unusual for the genus; the vesicle is commonly horizontal. After receiving the prostatic duct the ejaculatory duct continues through the penis, a simple conical projection into the small male atrium. The latter opens below by the male genital pore. Shortly behind this occurs the female genital pore, from which the muscular ciliated vagina extends dorsally, then curves posteriorly and downward to terminate where it receives the uteri (Fig. 1). A Lang's vesicle is absent as in the genus in general.

The color pattern and the erect prostatic vesicle serve to distinguish this species from others of the genus.

Distribution.—Region of Woods Hole, on wharves and pilings and along shores; also found by Verrill at other places in Vineyard Sound and in Long Island Sound, near New Haven, Conn., in dead shells of *Sycotypus* occupied by hermit crabs.

Stylochus ellipticus (Girard) 1850, new comb.

Syn. *Planocera elliptica* Girard 1850.
Stylochopsis littoralis Verrill 1873.
Stylochus littoralis (Verrill) Lang 1884.
Eustylochus ellipticus (Girard) Verrill 1892.

I am fortunately able to give a description of this species and establish its correct systematic position. There are available two vials of Verrill's collecting, a specimen in the Woods Hole material, and a number of individuals presented by Dr. Spieth and Mr. Burkenroad whom I here wish to thank for their trouble. Study of this material proves that

the animal is a typical member of the genus *Stylochus* and hence that Verrill was in error in erecting a new genus *Eustylochus* for the species (Verrill, 1892). *Eustylochus* thus becomes a synonym of *Stylochus*.

Description.—S. ellipticus (Fig. 2) is of oval or elliptical form and may reach a length of 20–25 mm.; specimens as small as 4 mm. were found to have the male system fully developed but the female system was immature. The body is flat but somewhat thick and opaque with undulated margins. The color is stated by Verrill and others to be various shades of cream, yellow, or brown, veined or reticulated with a lighter shade, or freckled with golden brown on a lighter ground color; the periphery is clear and translucent and there is usually a light cream to brown stripe in the posterior middorsal region. There are two nuchal tentacles filled with eyes; these are probably elongated and pointed in life but in the preserved specimens were rounded and somewhat sunk into depressions. Between and in front of the tentacles occur the cerebral eyes, subject to much variation. Not infrequently these occur in two pairs of groups of two eyes each (Fig. 2), but often there are three to five or more (up to nine were seen) eyes in each of the four groups and there may be a few scattered eyes farther back over the brain. Specimens have also been seen in which the cerebral eyes were not definitely arranged in four groups but formed a loose band on each side. A band of eyes occurs along the margin of the anterior third to half of the body; these eyes are larger in the mid-anterior region and diminish in size and number around the sides of the body. In many specimens no eyes could be seen along the margin of the posterior half or two-thirds of the body but in some individuals there are a few small eyes scattered along this region so that the entire margin in such cases is encircled with eyes.

The pharynx is of moderate size, smaller proportionately than in *Stylochus zebra* but well folded. The branches of the digestive tract radiate to the periphery making a reticulum through numerous anastomoses between adjacent branches.

Study of the copulatory apparatus in serial sections (Fig. 3) shows that the species is a typical *Stylochus* and that no grounds exist for separating it into a distinct genus. The genital pores are very close together near the posterior margin. The coiled vasa deferentia run alongside the pharynx and their distal ends turn dorsally and become muscular as they join the seminal vesicle proper. There is thus some development of the tripartite condition of the seminal vesicle seen in several species of *Stylochus,* but the central lobe is here much larger than the lateral lobes formed by the ends of the vasa deferentia. The central lobe tapers posteriorly into the ejaculatory duct which runs beneath the

prostatic vesicle to open into the penis lumen. The prostatic vesicle is a very large oval sac with a chambered interior and a thick muscular wall penetrated by the necks of extracapsular granule glands which discharge into the chambers of the interior. The prostatic vesicle has the horizontal orientation characteristic of the genus. Its duct joins the ejaculatory duct in the interior of the penis and the common duct so formed continues to the penis tip (Fig. 3). The penis papilla is a moderately muscular conical projection armed with a short stylet situated in the lumen. The occurrence of a stylet appears to be unusual in the genus *Stylochus*. The expanded male atrium closely surrounds the penis papilla and exits below by the male genital pore. The lining of the male atrium and the base of the penis papilla are filled with glandular secretion.

The large female pore, situated immediately behind the male pore, is lined by a very tall epithelium also having a glandular border of eosinophilous secretion. The vagina accompanied by cement glands proceeds dorsally and forward as a narrow tube which near the dorsal body wall turns sharply backwards and terminates by receiving the uteri.

Distribution.—Stated by Verrill to occur from New Haven, Conn., to Casco Bay, Maine, under stones in shallow water and tide-pools; not uncommon now at Cold Spring Harbor, Long Island, and along the shores of the west end of Long Island Sound where it joins the East River. Apparently scarce along the Connecticut shore, where four specimens were taken under a rock at Indian Neck, by M. D. Burkenroad. One specimen lent by Mr. Gray came from the bottom of a light-ship, off Woods Hole. Verrill's specimens from the Peabody Museum were as follows: one vial containing six animals, labelled Savin Rock (this is

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For all figures the numbers listed below have the same connotations.

1, marginal eyes, 2, tentacles, 3, tentacular eyes, 4, cerebral eyes, 5, brain, 6, pharynx, 7, vasa deferentia, 8, enlarged muscular terminations of the vasa deferentia, 9, seminal vesicle, 10, prostate vesicle, 11, glandular chambers of the prostate vesicle, 12, extracapsular granule glands, 13, penis papilla, 14, ejaculatory duct, 15, prostatic duct, 16, stylet, 17, male genital pore, 18, male atrium, 19, penis pocket, 20, female genital pore, 21, vagina, 22, cement pouch, 23, cement glands, 24, entrance of the uteri into the vagina, 25, uteri, 26, stalk of Lang's vesicle, 27, Lang's vesicle, 28, cirrus, 29, retractor muscle of the cirrus, 30, protractor muscle of the cirrus, 31, musculo-glandular fold of the vagina, 32, main intestine, 33, penis sheath, 34, female genital atrium, 35, granule glands, 36, common genital pore, 37, pouch from genital atrium.

PLATE I

FIG. 1. Copulatory apparatus of *Stylochus zebra*, sagittal view.
FIG. 2. *Stylochus ellipticus*, dorsal view of whole mount.
FIG. 3. *Stylochus ellipticus*, sagittal view of copulatory apparatus.

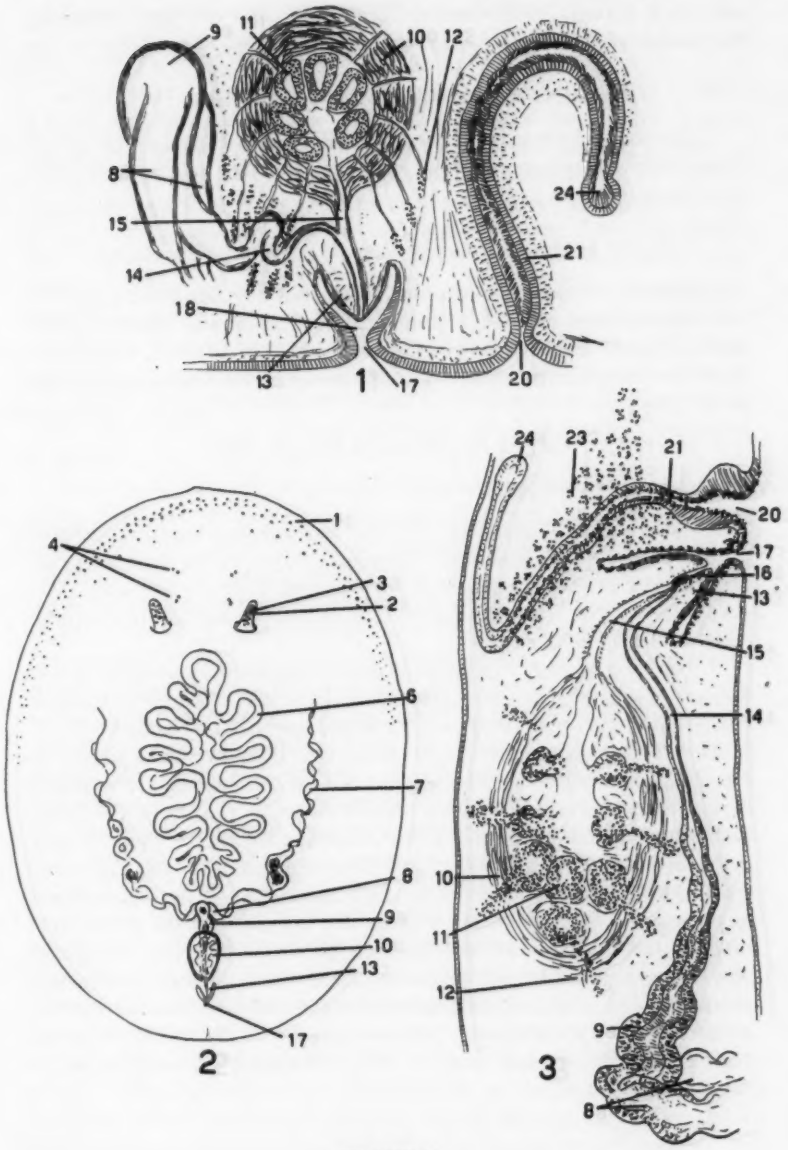
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PLATE I

near New Haven), under stones, Oct. 1871; and one vial containing one animal labelled *Stylochus littoralis,* New Haven, Oct. 1872.

Section Schematommata Bock 1913

Definition.—Acotylea without marginal eyes; commonly with paired cerebral and tentacular clusters of eyes close together and far back from the anterior margin.

Family Leptoplanidae Stimpson 1857 (emend. Bock)

Definition.—Schematommata with flat thin elongated bodies; prostatic vesicle, when present, interpolated; male apparatus directed backwards; definite tentacles usually absent but may be present; dorsal surface colored, generally some shade of brown; uteri confluent in front of the pharynx.

Genus Notoplana Laidlaw 1903

Syn. *Leptoplana* (in part).

Definition.—Leptoplanidae with true seminal vesicle and chambered prostatic vesicle into which the ejaculatory duct penetrates; male apparatus close behind the pharynx, far removed from the posterior margin; penis with or without a stylet; eyes in four clusters; tentacles generally absent or rudimentary.

It has been necessary to limit the genus *Leptoplana* into which it was formerly customary to throw almost any leptoplanid species to the first recognizable species ascribed to *Leptoplana,* namely *L. tremellaris* (O. F. Müller) 1774, and to other species which can be shown to be similar to this species. It unfortunately happens that the anatomy of *L. tremellaris* is somewhat aberrant and hence very few species remain in the genus *Leptoplana.* I know of none on the Atlantic coast of the United States but one occurs in Puget Sound and some may exist on the California coast although most of the Pacific coast species called *Leptoplana* must be transferred to other genera. With this limitation of the genus *Leptoplana,* the majority of the typical leptoplanids fall into the genus *Notoplana,* a genus of many species, all so much alike externally that identification is practically impossible without recourse to serial sections of the copulatory complex. Only one species of *Notoplana* is so far known from the Atlantic coast of North America but many occur on the Pacific coast.

NOTOPLANA ATOMATA (O. F. MÜLLER) 1776

Syn.? *Leptoplana variabilis* (Girard) Verrill 1892.

Description.—This species has already been well described by Bock (1913) and hence I shall be brief. The species, seen at Mt. Desert Island, Maine, may reach a length of 28 mm. and has the typical external aspect of the genus. The elongated oblanceolate body is broadest across the anterior fourth and then tapers to the rounded posterior end (Fig. 4). Young specimens are even more tapering than fully grown ones. The color is grayish brown above, more or less flecked and streaky. There are the usual four clusters of eyes, two rounded tentacular clusters representing the sites of the aborted tentacles and consisting of 6–10 large eyes and a few small ones; and the elongated cerebral clusters extending forward from the brain and composed of about 15–40 eyes of various sizes, but smaller than the largest of the tentacular eyes. The number of eyes increases with age so that young specimens have few eyes.

The whole anatomy is typically leptoplanid with a central elongated ruffled pharynx, radiating anastomosed digestive branches, uteri encircling the pharyngeal pocket, and vasa deferentia looping backwards across the stalk of Lang's vesicle (Fig. 5). The details of the copulatory apparatus have been discussed by Bock (1913) who presents a sagittal view and I give a ventral view (Fig. 5) made from a pressed live specimen. The vasa deferentia enter separately the angles of the rounded muscular seminal vesicle from which the ejaculatory duct penetrates more than halfway into the lumen of the spherical thick-walled chambered prostatic vesicle. From this the elongated curved penis pocket leads to the male atrium. A very small penis papilla next the prostatic vesicle bears the moderately long curved stylet which occupies the penis pocket and often protrudes from the male genital pore. There is a penis sheath. The wide muscular vagina continues beyond the entrance of the uteri forward, then bends backwards as a narrow stalk terminating in an enlarged Lang's vesicle (Fig. 5).

Bock is of the opinion that *Leptoplana variabilis* is identical with *Notoplana atomata*. While the former is undoubtedly a species of *Notoplana*, I find some points in Verrill's description of it (1892) which do not fit my observations on *N. atomata*. Thus Verrill gives the shape of *L. variabilis* as oblong or elliptical while *N. atomata* is always wedge-shaped, often markedly so, and describes the penis stylet as much longer and more coiled than I have seen it. I have not seen any other shape in a number of specimens examined than the simple bend figured.

Locality.—*Notoplana atomata* appears to be widely distributed along shores of the North Atlantic coast from Scandinavia to Maine; under stones, Mt. Desert Island, Maine, and probably northward.

Genus Euplana Girard 1893

Syn. *Discoplana* Bock 1913.

Definition.—Leptoplanidae of somewhat elongated form, without a prostatic vesicle; genital pores close together; tentacles lacking; penis unarmed, mostly small or even absent; Lang's vesicle present or absent. Type: *Euplana gracilis* (Girard) 1850.

I regret the necessity of making Bock's genus *Discoplana* a synonym of *Euplana* but the circumstances leave me no other course.

Euplana gracilis (Girard) 1850

Syn. *Prosthiostomum gracile* Girard 1850.
Prosthiostomum gracile Girard, Verrill 1892.
Euplana gracilis Girard 1893.

A number of specimens of a polyclad considered unnamed by members of the invertebrate staff at Woods Hole were secured in 1935. Study of serial sections revealed that the animal belonged to Bock's genus *Discoplana* and for some time the form was regarded as a new species of *Discoplana.* Then Verrill's figure of *Prosthiostomum gracile* Girard was recognized as identical with the polyclad in question (Verrill, 1892, p. 497). The question then arose whether the species actually is Girard's species, as Verrill thought. It is my opinion that there is very little doubt of this, although Girard was entirely mistaken in placing the animal in *Prosthiostomum.* Later, 1893, Girard recognized this error and created for the species the new genus *Euplana.* This genus appears to be quite valid under the rules and therefore I must regretfully declare *Discoplana* Bock 1913 a synonym of *Euplana* Girard 1893. The correct name of the animal is then *Euplana gracilis* (Girard).[1]

PLATE II

Fig. 4. *Notoplana atomata,* from life, dorsal view, Mt. Desert Island, Maine.
Fig. 5. Dorsal view of copulatory apparatus of *Notoplana atomata* as seen in live pressed specimen.
Fig. 6. *Euplana gracilis,* from life, dorsal view, Woods Hole, Mass.
Fig. 7. Another view of the anterior end of *Euplana gracilis,* from life.
Fig. 8. Sagittal section of the copulatory apparatus of *Euplana gracilis.*

[1] If the identity of the species under consideration with *Prosthiostomum gracile* be questioned, then *Discoplana* would again become a valid genus and the animal would be a new species of *Discoplana.*

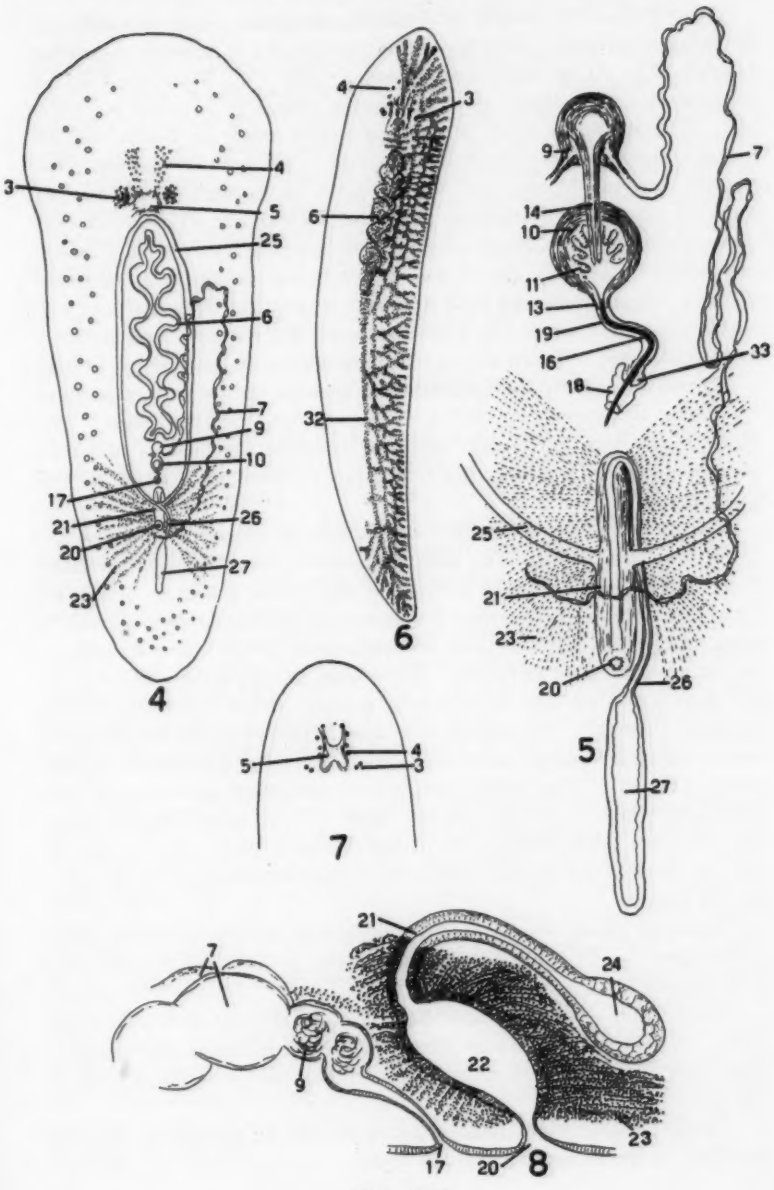
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PLATE II

Description.—E. gracilis is of slender elongated shape, resembling a fresh-water planarian (Fig. 6). The anterior end is at times somewhat pointed (Fig. 6), at other times rounded (Fig. 7). From the level of the brain the body tapers gradually to the obtuse or rounded posterior end. The species is small, with a maximum length of 8 mm. and of yellowish gray or brownish gray coloration. The eyes are the most distinctive external feature and readily serve to distinguish this species from all other North American polyclads. There are typically six eyes on each side, four in a lengthwise row, representing the cerebral clusters, and two eyes obliquely placed close together, representing the tentacular clusters. Specimens have been seen with some slight irregularity of eye number, such as one or two additional eyes, but the described arrangement is the rule. There are no marginal ocelli as supposed by Verrill.

The small, slightly ruffled pharynx is situated far forward, a position explaining the mistake in placing the species in *Prosthiostomum*. Behind the pharynx the median intestinal trunk is conspicuous in whole mounts (Fig. 6). From this trunk lateral branches extend to the periphery.

The main feature of the copulatory apparatus (Fig. 8) is the absence of the prostatic vesicle. The male and female genital pores are situated close together some distance behind the pharynx at about the middle of the body. Behind the pharynx the expanded thin-walled vasa deferentia unite to a but slightly muscular seminal vesicle which tapers directly to the male genital pore (Fig. 8). There is no penis papilla and no prostate vesicle nor are any extracapsular granule glands discernible in the available material. The female pore shortly behind the male pore leads into an expanded cement pouch into which open great numbers of cement glands (Fig. 8). The vagina then curves posteriorly and ventrally and terminates where it receives the two uteri. There is no Lang's vesicle. The uteri are confluent in front of the pharynx, then course along the sides of the pharynx behind which they expand considerably before joining the vagina.

E. gracilis closely resembles in its sexual anatomy several other species of *Euplana* (=*Discoplana*) differing chiefly in the total absence of a penis papilla.

Distribution.—Found in abundance in the Eel Pond at Woods Hole, Mass., on wharves and pilings among masses of hydroids and sponges; apparently formerly common in Boston Harbor and along the Connecticut shore.

Neotype.—One whole mount; paratype, one set of sagittal sections, deposited in the museum at Woods Hole.

Genus Stylochoplana Stimpson 1857 (emend. Bock)

Definition.—Leptoplanidae with true seminal vesicle and non-chambered prostate vesicle, the latter not penetrated by the ejaculatory duct; tentacles present or absent; eyes in paired cerebral and tentacular groups; separate or fused genital pores, which may be near the posterior margin.

In general aspect the species of this genus closely resemble those belonging to *Notoplana*. The essential difference between them lies in the prostate vesicle which is not chambered and not penetrated by the ejaculatory duct.

Stylochoplana angusta (Verrill) 1892

Syn. *Leptoplana angusta* Verrill.

[not *Stylochoplana angusta* (Verrill) Palombi 1928].

The finding at the Peabody Museum of a vial containing three specimens which are undoubtedly "*Leptoplana*" *angusta* has enabled me to determine the systematic status of this species. It was found to be a leptoplanid which fits best into the genus *Stylochoplana* although not entirely conforming to the present conception of this genus. It seems preferable, however, to expand the definition of the genus than to create a new genus for the species.

In 1928 Palombi identified a *Stylochoplana* species obtained by the Suez Canal expedition as *Leptoplana angusta*, and called it *Stylochoplana angusta* (Verrill). This identification is erroneous as the species in question differs decidedly from the latter. Professor Palombi has been informed of the necessity of creating a new specific name for his species.

Description.—*S. angusta* is stated by Verrill to be of elongated elliptical form, thin, with flexible undulated margins, rounded anterior end, and notched posterior margin. Figure 9 is a drawing of a whole mount made from one of Verrill's specimens; the other two were sectioned sagitally. The maximum length is given by Verrill as 12–16 mm., the breadth 4–6 mm., and the color as various shades of light brown. The cerebral and tentacular eyes form a continuous band on each side (Fig. 9), of which the posterior group of larger eyes on either side of the brain undoubtedly represents the tentacular eyes while the band of smaller eyes extending forward from these are the cerebral eyes. The narrow ruffled pharynx is somewhat posterior in position and this presumably accounts for the very posterior location of the copulatory complexes.

There is a large common genital pore close to the posterior margin shortly in front of the notch. This continues into a genital atrium which curves anteriorly receiving the vagina in its dorsal wall and the penis in its anterior wall. The small male copulatory complex (Fig. 10) conforms to that of the genus in general. The vasa deferentia after a union enter the anterior end of the elongated oval seminal vesicle. From this the curved ejaculatory duct runs to the prostatic vesicle. This is of oval form with a thin muscular wall and the interior lined by granule glands. No extracapsular granule glands were seen in the sections; naturally after sixty years in preservative the animals are not in very good histological condition. From the prostate vesicle, the ejaculatory duct continues backward and enters the small pyriform penis which projects into the male portion of the atrium as a slight conical eminence. The very long vagina runs from the roof of the atrium far forward and then after a short backward bend opens into a large spherical Lang's vesicle (Fig. 10). The uteri join the backward bend so that the stalk of Lang's vesicle is very short. The vagina is slightly muscular along its entire course and is accompanied throughout by cement glands. I am unable to determine from my material whether the uteri are confluent in front of the pharynx but Verrill's statements indicate that this is the case. In the whole mount, the uteri are much distended with eggs (Fig. 9) and extend anteriorly beyond the brain, a condition unusual in the Leptoplanidae.

The two sets of sagittal serial sections are identical except for one very puzzling feature. In one of them, the larger of the two, the common genital atrium continues dorsally behind the vaginal entrance as a wide pouch (Fig. 10), which appears to open on the dorsal surface, presumably into the posterior notch. The specimen is unfortunately imperfect at this place so that I cannot be sure of the actual presence of an opening there; but all appearances indicate that this is the case and I have so drawn the region in question (Fig. 10). The second specimen (Fig. 11) shows no trace whatever of such a pouch although it appears to be quite mature sexually. More material will be necessary to settle the matter of the occurrence and relations of the pouch in question. It may represent a ductus vaginalis, as in Bock's genus *Ceratoplana* (Bock, 1925).

Sty. angusta differs from the typical members of the genus in the far posterior location of the genital pore and the forward course of the vagina. The first point is not of so much importance since Bock (1924) found a Japanese species which he assigned to *Stylochoplana* and which also has a very posterior common genital pore. Kato (1937) reports

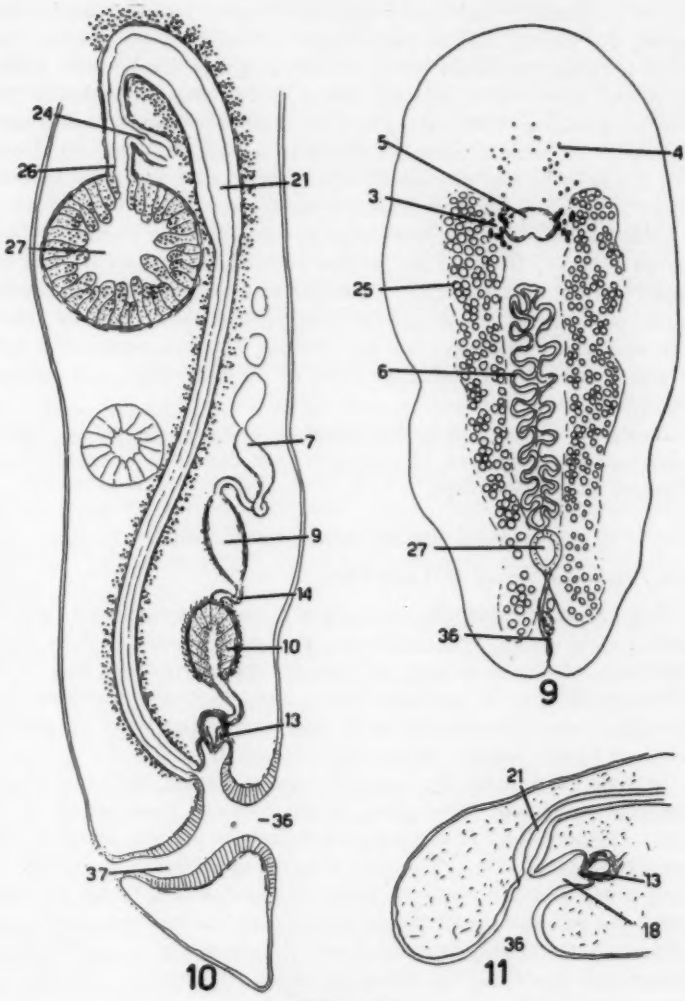


PLATE III

FIG. 9. *Stylochoplana angusta*, dorsal view of whole mount, neotype.

FIG. 10. Sagittal view of the copulatory apparatus of *Stylochoplana angusta*, from one of the sets of serial sections, showing problematical pouch from genital atrium dorsally.

FIG. 11. Terminal part of the copulatory apparatus of *Stylochoplana angusta*, from the second set of serial sections, showing absence of a pouch.

another Japanese *Stylochoplana* having the same feature. But in these species, the vagina, despite the posterior situation of the genital pore, makes the usual backward bend, so that Lang's vesicle lies well behind the genital pore. The situation found in *Sty. angusta* with the long anterior extension of the vagina and the location of Lang's vesicle much anterior to the male apparatus appears to be unique. One could consider it a basis for the erection of a new genus but I prefer to leave the species in *Stylochoplana,* at least for the present.

Distribution.—*Stylochoplana angusta* was found by Verrill at Provincetown, Mass., 1879, on the bottom of a whaling vessel recently arrived from the Carolina coast. The polyclad probably came from this region, especially as it was associated with other animals said by Verrill to be southern. The vial from the Peabody Museum contained a label on which was printed: Off Cape Cod, U. S. F. C. 1879, and written: Leptoplana, vessel bottom.

Neotype.—Whole mount designated as neotype; one set of sagittal serial sections, designated as paratype; both deposited in the Peabody Museum, Yale University.

Genus Hoploplana Laidlaw 1902

Syn. *Planocera,* Group B, Lang 1884.

Definition.—Schematommata without a true seminal vesicle or penis papilla; ends of the vasa deferentia enormously enlarged into thick-walled muscular tubes serving as accessory seminal vesicles; penis stylet fastened directly to the prostate vesicle; tentacles present; with cerebral and tentacular eye clusters, the latter closely embracing or in the tentacle bases; no Lang's vesicle; shape oval or rounded.

In 1902 Laidlaw rightly removed some of Lang's species of *Planocera* to a new genus *Hoploplana,* which does not even belong to the family Planoceridae. It also does not entirely fit into the family Leptoplanidae, so that Bock (1913) left it as an appendix to the latter. It would seem to me desirable to place *Hoploplana* in a family by itself despite the fact that its affinity to the Leptoplanidae is indicated by the finding in Puget Sound of a species of *Notoplana, N. segnis* Freeman 1933, which has also very enlarged, highly muscular terminations of the vasa deferentia.

Hoploplana grubei (Graff) 1892

Syn. *Planocera grubei* Graff 1892.

This species, common on the Sargassum, has been adequately described and figured by von Graff (1892). Three specimens, brought

to me for identification by Dr. Hadley of the Invertebrate staff at Woods Hole, were taken from the Sargassum in Vineyard Sound, August 3, 1938. The coloration was stated to consist of gray reticulations on a pinkish-brown or flesh ground. M. D. Burkenroad of the Peabody Museum, who has collected a number of specimens of this species from the Sargassum in the Gulf of Mexico and North Atlantic, reported the color as consisting of livid white reticulations on a brown ground. It is clear that von Graff's conception of the color, based on preserved material, is erroneous. Preserved specimens give no idea of the original coloration. A colored figure of this species made by Dr. J. F. G. Wheeler of the Bermuda Biological Station is being published in my article on the polyclads of Bermuda and the Sargassum (Hyman, 1939).

Hoploplana inquilina (Wheeler) 1894

Syn. *Planocera inquilina* Wheeler 1894.
Hoploplana inquilina (Wheeler) Bock 1913.

This species, familiar at Woods Hole, was rightly removed by Bock to the genus *Hoploplana*. Wheeler presents an excellent figure of the entire animal but his account of the internal details leaves much to be desired. I have therefore deemed it necessary to make a study of serial sections.

Description.—The expanded thin-walled vasa deferentia packed with sperm course alongside the pharynx near the posterior end of which they alter into thick-walled muscular accessory seminal vesicles (Fig. 12). These, after reaching a level behind the male pore narrow, approach the median line, and fuse to a narrow duct, the common vas deferens. This runs anteriorly penetrating into a round somewhat muscular organ which Wheeler considers the penis. It is, in fact, the prostatic vesicle and this mistake accounts for Wheeler's inability to find such a vesicle. It is not, to be sure, a very typical prostate. It has an excessively thick wall, containing muscle fibers coursing parallel to the surface contour and in the center a small area of granule glands radiating around the central small ejaculatory duct. The granule glands are best seen in frontal section (Fig. 12) as they are oriented parallel to such a section. Scattered glands also seem to extend into the thick wall of the prostate vesicle. After passing through the glandular region the ejaculatory duct terminates in the base of the stylet which constitutes the entire penis (Fig. 13). It is directed ventrally in the available specimens, extending into the male atrium. The latter has quite a muscular wall and is lined by an extremely tall columnar epithelium which bulges from the genital pore (Fig. 13).

It will be seen from the foregoing description that the male apparatus of *H. inquilina* accords completely with that found in other members of the genus. The vasa deferentia form accessory seminal vesicles, there is no true seminal vesicle, and the prostate vesicle bears directly the penis stylet without the intervention of any penis papilla proper.

The female copulatory apparatus consists simply of the vagina which extends dorsally from the genital pore receiving the secretion of the thickly strewn cement glands. The vagina is somewhat muscular, and lined by tall slender epithelial cells. After making a backward bend, the vagina constricts and beyond this constriction the shell glands cease and the lining epithelium alters to large vacuolated cells. This portion of the vagina resembles histologically a Lang's vesicle but morphologically is not such since the uteri open into its ventral end (Fig. 13).

Distribution.—Hoploplana inquilina has been recorded only from the vicinity of Woods Hole where it inhabits the mantle cavity of the large gasteropod *Sycotypus*.

Family Planoceridae Lang 1884 (emend. Bock 1913)

Definition.—Schemattommata with a cirrus instead of a penis; cirrus lined with spines, hooks, or ridges eversible to the exterior; with tentacles; with tentacular and cerebral eyes; copulatory complexes immediately behind the pharynx; with true or accessory seminal vesicles; prostate vesicle free or interpolated.

Genus Gnesioceros Diesing 1861 [2]

Syn. *Pelagoplana* Bock 1913.

Definition.—Planoceridae with wedge-shaped pellucid bodies; with tentacles containing eyes; cerebral eyes also present; with a true seminal vesicle and interpolated prostatic vesicle; cirrus armed with parallel toothed bands; vagina with a powerful musculo-glandular fold; Lang's vesicle transverse. Type, *Gnesioceros sargassicola* (Mertens) 1833.

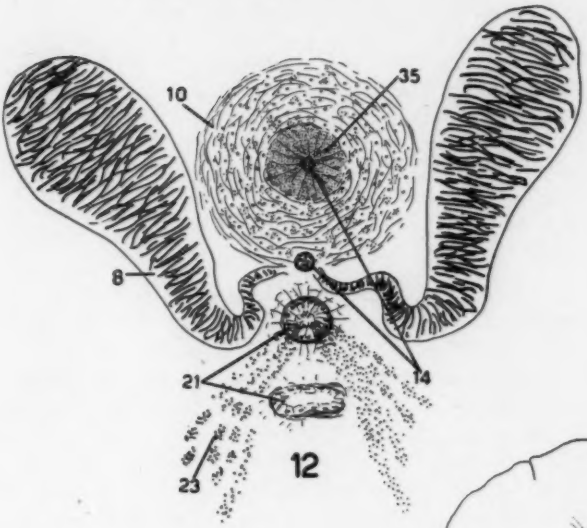
PLATE IV

Fig. 12. Frontal view of the vasa deferentia and prostate vesicle of *Hoploplana inquilina*, showing area of granule glands in the center of the vesicle around the ejaculatory duct.

Fig. 13. Sagittal view of the copulatory apparatus of *Hoploplana inquilina*.

Fig. 14. *Gnesioceros verrilli*, dorsal view, whole mount, Woods Hole specimen.

[2] In 1861 Diesing created the genus *Gnesioceros* for two species: *"Planaria" pellucida* and *"Planaria" sargassicola* both described by Mertens in 1833. *Planaria pellucida* belongs to the genus *Planocera* and was removed to that genus in 1844 by Oersted. Diesing did not indicate which of the two species he considered typical of the genus *Gnesioceros*. I hereby remove *pellucida* from the genus and declare *sargassicola* to be the type of *Gnesioceros*, thus making the genus valid.



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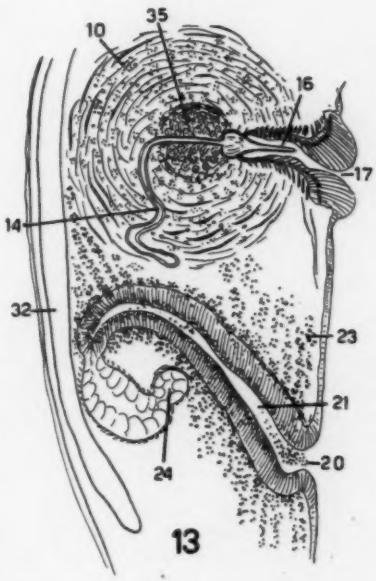

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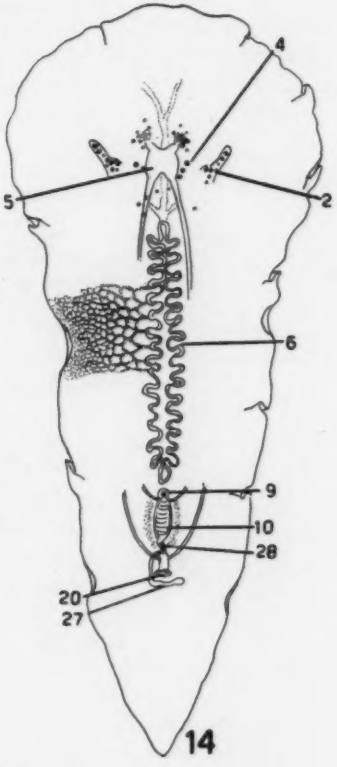

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PLATE IV

Gnesioceros sargassicola (Mertens) 1833

Syn. *Planaria sargassicola* Mertens 1833.
Stylochus sargassicola Ehrenberg 1836.
Planocera sargassicola Oersted 1844.
Stylochus pelagicus Moseley 1877.
Gnesioceros mertensi Diesing 1861.
Stylochus sargassicola (Mertens) Lang 1884.
Stylochoplana sargassicola (Mertens) Graff 1892.
Pelagoplana sargassicola (Mertens) Bock 1913.

This is the common polyclad of the Sargassum found on the floating weed in various parts of the world, common in the Gulf of Mexico, Caribbean, and North Atlantic. I took several specimens alive from the Sargassum at Bermuda and Burkenroad collected hundreds of specimens during an expedition to study the biology of Sargassum inhabitants. Alive the animal is pellucid with small brown spots and an expanded anterior end tapering to a pointed posterior end. It has been figured and described by von Graff (1892) and I have given an account of the structure of the cirrus (Hyman, 1939). A specimen of this species was taken from the Sargassum in Vineyard Sound, August 3, 1938.

Gnesioceros verrilli, New Name

Syn. *Imogine oculifera* (Girard) Verrill 1892.
(not *Imogine oculifera* Girard 1853).

In 1882 Verrill found in Quisset Harbor a single specimen of a polyclad which he assigned to Girard's species *Imogine oculifera*. This identification is clearly erroneous since Girard plainly states that *Imogine oculifera* has a band of eyes completely encircling the margin and hence is a stylochid while Verrill's figure plainly shows a planocerid. Two vials of specimens were obtained from Mr. Gray which I am confident are identical with Verrill's specimen. Examination of these animals proved that they belong in the genus *Gnesioceros*. As it is necessary to give a specific name to Verrill's species, I propose to call it *verrilli*.

Description.—*G. verrilli* is very similar in all respects to *G. sargassicola*. The graceful body is anteriorly expanded (Fig. 14), being widest at about the level of the brain, and from there tapers gradually to the pointed posterior tip. The species is small, around 6–8 mm. in length. The preserved specimens were colorless but Verrill gives the color as carmine red. As this color was stated, however, to be limited to the intestinal branches, it is nearly certain that it resulted from

ingested food and that the animal is in fact, like *G. sargassicola,* pellucid with small brownish marginal spots. There are two elongate nuchal tentacles, each containing several eyes, most of which are in the tentacle bases. Between the tentacles, extending some distance behind and in front of them, are two loose elongate groups of cerebral eyes, of about 8–12 eyes each (Fig. 14). From the long, narrow, slightly ruffled pharynx a rich net of anastomosing intestinal branches radiates to the periphery.

The copulatory complexes behind the pharynx are very similar to those of *G. sargassicola* which have been described by Graff, 1892, and Hyman, 1939. The vasa deferentia enter separately the ventral angles of the pyriform seminal vesicle (Fig. 15) which ascends and narrows to the ejaculatory duct opening into the anterior end of the prostate vesicle. The latter is an elongate structure divided into chambers by cross-partitions and covered with a thick muscular coat which operates the cirrus. The prostate vesicle continues directly into the cirrus, a curved beak-like structure composed of hard yellow material, presumably not chitin but an albuminoid. The structure of the cirrus was described and figured in another place (Hyman, 1939) and hence a brief statement will be made here. The cirrus possesses a median groove and thus resembles a conch shell. It is made of parallel toothed bands (Fig. 16) which can be pulled into the interior along the groove by the action of the retractor muscle fastened along one side of the prostate vesicle. The protractor muscle on the other side of the prostate vesicle is attached to the central part of the bands. When it contracts, it pulls the bands to the outside where they take a horizontal course. The cirrus is situated in a pocket which continues into the male atrium and so to the male genital pore.

The female genital pore is shortly behind the male pore. From it the ciliated, slightly muscular vagina extends dorsally, then curves backward and ventrally and receives the uteri. Beyond this union the female canal continues as the stalk of Lang's vesicle and opens into the latter. Lang's vesicle is unique in the genus *Gnesioceros* in taking a transverse course, so that it embraces the rear and sides of the female genital pore in a somewhat crescentic shape. The genus is further notable for the presence in the vagina of a powerful musculo-glandular fold, consisting of a heavy muscle mass to which are attached numerous gland cells. In *G. sargassicola* this fold is ring-shaped, completely encircling the vagina; but in *G. verrilli* it is well-developed only in the anterior wall of the vagina, the side facing the cirrus, and but slightly present and more diffuse in the posterior vaginal wall (Fig. 15). The epithelium over the main muscle mass is reduced to a cuticularized membrane. In *G. sar-*

gassicola the vaginal epithelium from the musculo-glandular fold to the genital pore is also cuticularized and thrown into lengthwise ridges; but this is not the case in *G. verrilli*.

These differences in the vaginal structure constitute the chief distinctions between *G. sargassicola* and *G. verrilli*. Other differences are: the pharynx appears to be narrower and less ruffled in *G. verrilli* than in *G. sargassicola* and the prostate vesicle narrower and longer relative to the cirrus region in the former than in the latter.

Neotype.—It has seemed desirable to designate a neotype in the form of a whole mount deposited in the museum at Woods Hole; and a set of serial sections is also presented to this institution.

Distribution.—Taken from the eel grass, Devil's Foot Island, Woods Hole, Mass., December, 1930 and October 28, 1931; also found by Verrill in Quisset Harbor, Buzzard's Bay, on sandy bottom in four or five fathoms, September 4, 1882.

Suborder Cotylea Lang 1884

Definition.—Polyclads with a sucker behind the female genital pore (a few exceptions); pharynx ruffled to tubular; copulatory complexes generally in the anterior half of the body; prostatic vesicle, when present, always free; uteri generally behind the female genital pore; tentacles when present of the marginal type; eyes on the anterior margin, on the tentacle bases when these are present.

Family Eurylepidae Lang 1884

Definition.—Cotylea with anteriorly directed tubular pharynx; male apparatus below or immediately behind the pharynx, directed forward, with a free prostate vesicle and armed with a stylet; with or without uterine glands; tentacles vary from well-developed pointed marginal folds to none; with paired cerebral clusters of eyes and paired marginal clusters which are on the tentacles when these are present.

Genus Eurylepta Ehrenberg 1831 (emend. Lang 1884)

Definition.—Eurylepidae with well-developed pointed tentacles; main intestine with very few (up to about five) lateral branches; male

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PLATE V

Fig. 15. Sagittal view of the copulatory apparatus of *Gnesioceros verrilli*.

Fig. 16. Prostate vesicle and cirrus of *Gnesioceros verrilli*.

Fig. 17. Tentacles and eye distribution of *Eurylepta maculosa*, specimen collected by Verrill.

Fig. 18. Sagittal section of the same specimen as Fig. 17, showing pharynx and female copulatory apparatus; because of damage the male apparatus, to be expected immediately behind the pharynx, is missing.

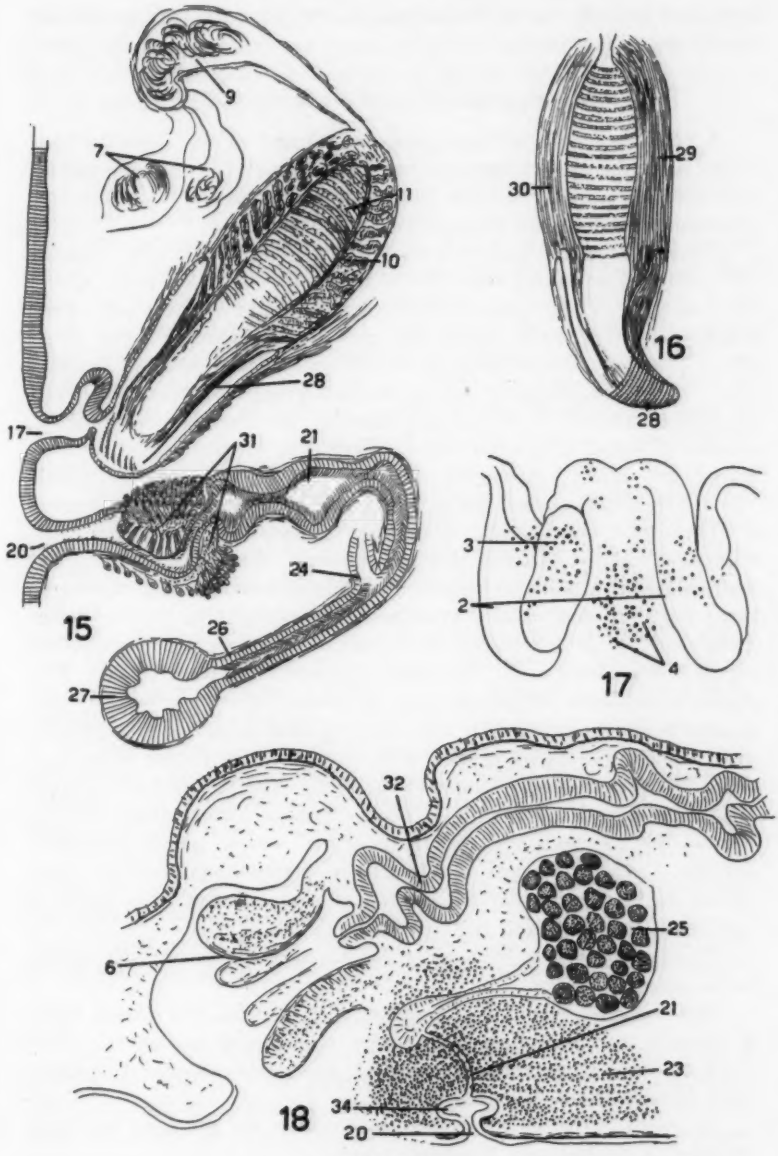
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PLATE V

apparatus beneath the posterior end of the pharynx; uterine glands mostly one pair or none.

EURYLEPTA MACULOSA VERRILL 1892

A single specimen of this species was found at the Peabody Museum; after the eye arrangement had been drawn (Fig. 17) the animal was cut into sagittal sections but these were not very satisfactory. However, it seems desirable to describe the findings.

Description.—The species is said by Verrill to have a thin, changeable, elliptical or oblong body, 10–12 mm. long, with thin often undulated margin. The anterior margin is upfolded to make two fairly long bluntly pointed tentacles which bear eyes on their anterior faces (Fig. 17). There are, as also noted by Verrill, no eyes at or near the tentacle tips. There are elongated paired cerebral groups of eyes which are continued forward to the margin between the tentacles by scattered clusters of eyes. The preserved specimen was distinctly mottled with brown and white; Verrill gives the color as pale, yellowish or pinkish white, irregularly specked and mottled or veined with purplish or brown.

The serial sections show a typical euryleptid structure (Fig. 18) with short tubular pharynx directed forward. From the pharynx the main intestinal trunk runs backward in the middorsal line and has about three pairs of wide lateral branches, thus conforming to the genus *Eurylepta.* To either side of the intestinal trunk is a large thin-walled uterus stuffed with eggs. No uterine glands have been found. The region immediately behind the pharynx where the male copulatory apparatus would be expected is unfortunately broken and no trace of the male copulatory apparatus has been found. The female apparatus (Fig. 18) appears to be completely present. The female pore, shortly behind the pharynx, leads into a small expanded atrium from which a narrow vagina, receiving the innumerable cement glands, proceeds dorsally. The great mass of the cement glands almost obscures the female duct. The vagina widens into a small chamber from which a duct runs posteriorly and receives the uteri.

There appears to be little doubt that the species was correctly placed in the genus *Eurylepta.*

Distribution.—Found by Verrill on piles, in mud, and among algae at low tide at Woods Hole and vicinity, stated to be uncommon. The Peabody Museum specimen was labelled: *Eurylepta maculosa* V. cotype, mud, Woods Hole, Aug. 2nd.

Specimen.—The set of sections made from the specimen has been returned to the Peabody Museum.

Whole mounts or preserved specimens of most of the species described in this paper (except those belonging to the Peabody Museum) have been deposited in the Museum at Woods Hole. In some cases a set of serial sections has also been presented to this institution.

Summary

1. *Stylochus zebra* (Verrill) 1882 is shown to have been correctly placed by Verrill in the genus *Stylochus*.

2. *Eustylochus ellipticus* (Girard) 1850 is shown to be a typical member of the genus *Stylochus*, its name thus becomes *Stylochus ellipticus*, and *Eustylochus* becomes a synonym of *Stylochus*.

3. *Notoplana atomata*, usually erroneously called *Leptoplana variabilis*, is the most common polyclad of the Maine coast.

4. *Prosthiostomum gracile* Girard 1850 has been rediscovered at Woods Hole. The later name *Euplana gracilis* which Girard gave to this species is valid and *Discoplana* Bock 1913 becomes a synonym of *Euplana* Girard 1893.

5. Specimens of *Leptoplana angusta* Verrill 1892 have been found at the Peabody Museum, Yale University. The species is placed in the genus *Stylochoplana* although it differs somewhat from the typical members of the genus. The name is then *Stylochoplana angusta* (Verrill).

6. As noted by Bock (1913), *Planocera inquilina* Wheeler 1894 must be transferred to the genus *Hoploplana* and the correct name of the species is *Hoploplana inquilina*. What Wheeler considered to be the penis is in reality the prostatic vesicle.

7. Two common and well-known Sargassum polyclads have been taken on the Sargassum in Vineyard Sound; these are *Hoploplana grubei* (Graff) 1892 and *Gnesioceros sargassicola* (Mertens) 1833. *Pelagoplana* Bock 1913 becomes a synonym of *Gnesioceros* Diesing 1861.

8. Another species of *Gnesioceros*, *G. verrilli*, new name, occurs around Woods Hole; it was seen by Verrill in 1882 and erroneously identified by him as *Imogine oculifera*.

9. A single specimen of *Eurylepta maculosa* Verrill 1892 was found at the Peabody Museum; some details of its anatomy are described.

Addendum

Since this manuscript was sent to press there have appeared three articles on Atlantic coast polyclads by A. S. Pearse and associates (*Proc. U. S. Nat. Mus.*, 86, *Jour. Elisha Mitchell Sci. Soc.*, 54, and *Bull. Mt. Desert Is. Biol. Lab.* 1938). I am unable to agree with many of the identifications, taxonomic placing, and anatomical findings in these

articles but detailed criticism will be withheld until a study of Pearse's specimens now under way has been completed.

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EFFECTS OF COLCHICINE ON THE CLEAVAGE OF THE FROG'S EGG (RANA PIPIENS)

DOROTHY M. KEPPEL AND ALDEN B. DAWSON

(*From the Biological Laboratories, Radcliffe College, and Harvard University*)

The most striking biological effect of the alkaloid drug, colchicine, is its capacity, when applied in the proper concentration, to arrest mitosis in the metaphase (Dustin, 1934, 1935). Under certain conditions, especially in plants, this drug has also been shown to be effective in producing polyploidy, doubtlessly due to its ability to inhibit to a greater or lesser degree the orderly sequences of the mitotic process (Blakeslee and Avery, 1937; Nebel and Ruttle, 1938).

There have been a great many studies of the effect of physical and chemical changes in the external environment on the developing egg of the frog. Some of the early results obtained, especially by centrifuging (O. Hertwig, 1897), and by exposure to high temperatures (O. Hertwig, 1898), rather closely parallel the effects produced by treatment with colchicine. In the present report the observations are limited to the changes induced by relatively short exposures to the drug applied almost immediately after fertilization.

Methods

The eggs of *Rana pipiens* were obtained and inseminated by the method of Rugh (1934). After a quarter of an hour the eggs were flooded with water and in another quarter of an hour were separated by dissection but the jelly was not removed. They were then put into the colchicine solutions. In later experiments the solutions were made up and used in the dark, except for a red light, since colchicine is affected in solution by light (Lits, 1936). The experiments were conducted at 8° and 20° C.

Description

In the first series of experiments, the colchicine solutions were made up in concentrations ranging from 1 : 1000 to 1 : 1,000,000 and the eggs were left in the solutions continuously. In the 1 : 1000 solution all cleavage activity was suppressed, and eventually disintegration set in. In the 1 : 10,000 solution the first cleavage took place as in the controls, the second cleavage set in at the normal time but showed some irregularities.

Soon after this, the membranes of most of the eggs seemed to be tightly stretched, appearing as though an abnormal amount of water had been absorbed. The cleavage grooves became more and more shallow. No further cleavage took place, and four and a half hours after fertilization no evidences of cleavage were visible except the presence of light streaks on the animal hemisphere. These streaks, which were due to the absence of superficial pigment, coincided with the position of the cleavage furrows. They appeared to have been caused by the movement of pigment deeper into the egg in association with the development of the clefts.

In the 1 : 100,000 solution of colchicine, the first, second, and third cleavages frequently took place as in the controls. Shortly after the initiation of the third cleavage, many of the eggs showed diminished grooves, the others continued dividing as in the controls. On the following day, some of those in which the grooves were fading showed no trace of cleavage except the light streaks, while others had the animal pole region largely cellular with the vegetative hemisphere uncleaved. The proportion of cellular to non-cellular material varied considerably. These were all dead on the third day.

In the 1 : 1,000,000 solution, the early cleavage was like that in the controls, no fading being evident except possibly on the vegetative halves. The second day embryos varied from completely normal to those with a small cell cap in the region of the animal pole, the rest of the egg being uncleaved. These embryos also, even those that appeared normal, were dead on the third day.

It is evident from these preliminary experiments that the eggs varied considerably in their susceptibility to colchicine, and that with a continuous exposure it was relatively toxic. A 1 : 100,000 exposure for one hour was found to give a fair number of abnormal forms without too high mortality and even a concentration of 1 : 10,000 for the same length of time was also satisfactory, although the mortality was somewhat greater. Most of the eggs in both these concentrations gave rise to normal embryos. The following studies, then, were made on selected individuals showing the characteristic abnormalities.

It is extremely difficult to interpret fully many of the abnormalities obtained by the treatment with colchicine. It was not possible to secure an orderly and progressive series of developmental stages as in normal development. The wide range of susceptibility of the eggs to the drug and the diverse effects obtained make it necessary to consider each egg individually and attempt to relate the conditions in earlier cleavages to the morphological patterns found in the more advanced embryos. In general the segmentation of treated eggs becomes meroblastic, with the

cap of cells produced in the region of the animal pole resting upon a more or less completely undivided yolk.

Early Cleavage

Reference has already been made to the fading of cleavage. Observations on living eggs and sections show that it takes place first at the vegetative pole, and sometimes a furrow extending halfway down the egg is left (Figs. 1, 2, and 3), while in other cases no trace of the cleavage remains, or only a shallow depression may persist at the surface of the animal pole. In some in which no external groove is left a cleft inside the egg can be seen in sections. In rare instances the first cleavage may persist even on the vegetative hemisphere, though subsequent cleavages are restricted in their persistence to the animal hemisphere. The nuclei may go on dividing although there is no cytoplasmic division. In several cases in which the early cleavage furrows had completely disappeared, a curious sort of delayed or secondary cleavage appeared at about the time when the controls were in the blastula stage (three days at 8° C.). In such cases a few relatively small cells were outlined on the surface, and contained one or more large vacuolated nuclei. More similar nuclei appeared in the main body of the egg.

The significant result of the arrest or retardation of the early mitosis and the secondary fading of the early cleavage furrows, especially in the vegetative hemisphere, is the production of an early blastula in which definitive cells tend to be restricted to the animal hemisphere. The degree of restriction of cell cleavage is variable. In extreme cases only a small cap of cells is formed but all gradations from this condition to almost complete cleavage of the yolk of the vegetative half may be encountered.

Blastulae

In many embryos with only a small cellular area in the region of the animal pole a stage comparable to the blastula is not attained. The cells divide progressively but do not assume any regular pattern. They are usually loosely grouped above the undivided yolk mass without the formation of a definite segmentation cavity (Figs. 4 and 6). This condition was observed most frequently in embryos which had developed at the lower temperature, 8° C.

In others, a modified blastula, consisting of a cellular roof which is separated more centrally from the uncleaved yolk by a segmentation cavity or blastocoele, is formed. This cavity apparently may be secondarily enlarged by the progressive vacuolation of the adjoining yolk mass (Fig. 7). Below the cavity are the free nuclei of the yolk, becom-

ing larger, more abnormal, and more irregularly spaced toward the vegetative pole. They are usually surrounded by dense accumulations of pigment.

In embryos in which approximately half of the material is cellular the blastula is frequently more complete with a cellular floor of variable thickness. The roof is often differentiated into two layers comparable to the outer, epidermal and inner, nervous layer. Occasionally, however, part of the animal half may fail to undergo segmentation while a considerable amount of the yolk is cleaved. In such cases the degree of primary segmentation that may have occurred in the yolk is difficult to determine. A study of living animal-hemisphere embryos showed that the first two cleavages could often be seen on the surface of the vegetative pole and subsequently faded. Whether or not these cleavages

EXPLANATION OF PLATE

Figures 1 to 9 are photomicrographs taken at several magnifications. The magnification was varied in order to bring out significant details or to include sufficient area to show relationships. In all instances the solutions of colchicine were applied for one hour, beginning thirty minutes after insemination.

PLATE I

Explanation of Figures

Figs. 1 and 2. Modification of early cleavage; 4 hours, 20 minutes after insemination; temperature 20° C.; colchicine, 1 : 10,000.

Fig. 3. Early cleavage; 5 hours, 20 minutes after insemination; temperature 20° C.; colchicine, 1 : 10,000. The plane of section is oblique to the polar axis giving a false impression of the degree of cleavage of the vegetative hemisphere.

Fig. 4. External view of late cleavage showing restriction of cleavage to the animal half of the egg; 72 hours after insemination; temperature 8° C.; colchicine, 1 : 100,000.

Fig. 5. Partial embryo; 48 hours after insemination; temperature 20° C.; colchicine, 1 : 100,000. From sections it appears that the first cleavage persisted and then cell division was restricted in one blastomere more than in the other.

Fig. 6. Late atypical cleavage; 72 hours after insemination; temperature 8° C.; colchicine, 1 : 100,000. Cleavage is sharply restricted to a cap of cells in the region of the animal pole. Large, numerous yolk-nuclei with concentrations of pigment about them are scattered through the uncleaved yolk.

Fig. 7. Animal-hemisphere blastula; 48 hours after insemination; temperature 20° C.; colchicine, 1 : 100,000. Only the roof of the blastocoele is cellular. The floor is composed of unsegmented yolk which is highly vacuolated. The cellular configuration (on the right) possibly represents the beginning of gastrulation by involution.

Fig. 8. A transverse section of the upper half of an abnormal gastrula; 48 hours after insemination; temperature 20° C.; colchicine, 1 : 100,000. Neural plate, notochord, mesoderm and an incomplete layer of entoderm are present. The underlying yolk is undivided and ventrally an area of degeneration is seen.

Fig. 9. An area from the cellular roof of a blastula; 48 hours after insemination; temperature 20° C.; colchicine 1 : 100,000. The nuclei of this embryo are of two distinct sizes. The smaller are of normal size. The larger may be due to tetraploidy.

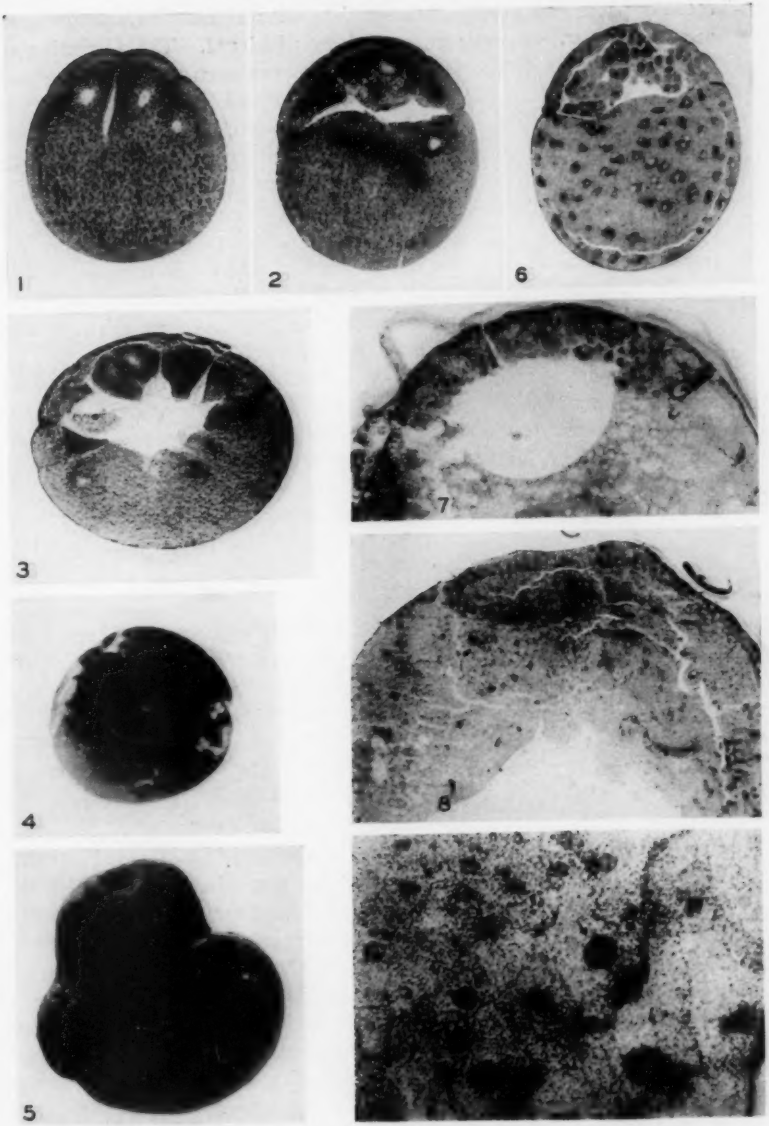

PLATE I

actually passed completely through the yolk was not determined, but it seems fairly safe to conjecture that they had not. The formation of the cellular floor of the blastocoele may have occurred simultaneously with cleavage of the cells nearer the animal pole but there is evidence that in many cases the cleavage of the yolk occurs secondarily, probably through the progressive organization of cells from a region comparable to the germ-ring of normal embryos. As far as it is possible to judge from sections, the process of cellulation occurs around free nuclei directly adjacent to previously formed cells. In these regions the free nuclei are more likely to be normal in size and shape than those at a greater distance from the zone of definitive cells, i.e., nearer the vegetative pole. The cells formed are at first more or less spherical and sometimes extracellular yolk remains between them.

In many instances there was considerable disturbance of the distribution of pigment especially at the margin of the polar cap. Frequently the polar pigment was carried down as irregular streamers over the vegetative hemisphere leaving an irregular border between the animal and vegetative halves. In other instances the local disappearance of pigment on the surface of the animal hemisphere appeared to be due to its being carried deeper into the egg. Degenerative changes may be involved.

Gastrulae

It seems probable in the embryos in which segmentation is restricted to a cap of cells at the animal pole, that gastrulation could not occur. However, even in these, there is a tendency for the marginal cells of the cap to show a regional separation from the uncleaved yolk suggesting the initiation of gastrulation. In more complete blastulae, gastrulation may set in between the cleaved and uncleaved material. More often the yolk is cellular in the immediate region of the invagination. However, the evidence on the process of gastrulation is not conclusive. Externally the blastopore lip is straight and does not seem to be extended laterally very far around the circumference of the egg. In cases in which the yolk is not cleaved at the point of gastrulation the process seems to be limited to simple involution with the cells migrating to form a more or less continuous layer under the roof of the blastula. These cells are usually comparatively small and heavily pigmented, resembling those of the superficial epidermal layer. There does not seem to be any superficial down-growth in the region of gastrulation but laterally and anteriorly there is considerable overgrowth of a single layer of epidermal cells over the undivided yolk.

In embryos with cleaved yolk at the point of gastrulation there is usually a sac-like invagination which extends progressively into the blas-

tocoele and rests on the upper surface of the undivided yolk. In such cases the blastocoele is eventually obliterated and a small archenteron is established.

Later Development

Many embryos die after the initiation of the modified gastrulation, probably because of their inability to carry the process to any degree of completion. However, several survived in which gastrulation appeared to be represented primarily by involution. In these a disc-like blastoderm was developed which consisted of a neural plate with rudimentary neural folds, notochord, tissue occupying the position of dorsal mesoderm and a thin, sometimes incomplete layer of underlying entoderm (Fig. 8). A careful study of serial sections fails to yield any evidence of invagination. Caudally the epidermal cells, forming a free margin, rested directly upon the uncleaved mass of yolk. The yolk-filled tissue lateral to the notochord is interpreted as being derived by the progressive cellulation of the yolk adjacent to its junction with the margin of the blastula roof. The cells of the notochord and entoderm were relatively small and heavily pigmented. This, however, does not constitute an irrefutable argument for their origin by involution as the distribution of pigment is very irregular in these abnormal embryos. Furthermore, even in normal embryos the cells of this region contain considerable pigment, possibly related to their metabolic activity and more rapid multiplication.

Usually the segmentation of the animal hemisphere occurs uniformly and the partial embryos tend to be symmetrical. In some cases, however, the restriction of cleavage is unequal and asymmetrical embryos are produced. Such an embryo is illustrated in Fig. 5. It appears that in this case the first cleavage persisted and then the cell division of the region of the animal pole on one side was restricted more than on the other. Embryos do not survive beyond the neural fold stage.

Nuclear Size

In about half of the animal hemisphere embryos cells were found that were much larger than normal. Those generally occurred in groups and might be either epidermal or deeper-lying cells. They had correspondingly large nuclei (Fig. 9). Mitoses were observed but they were seldom favorable for chromosome counts due to the dense concentration of pigment granules about the spindle figure. Polar views adequately demonstrated that the number of chromosomes in the large cells was greater than normal but the exact ratio could not be established. It would seem quite probable that these represent tetraploid conditions. In

several embryos, one in particular, sectioned at late cleavage, the cells were of varied sizes, and the mitoses were very irregular. Eccentric, distorted monasters often at the sides or corners of the cells were frequent. Such conditions probably explain the variation in nuclear size.

In some cases, especially in eggs which had developed at 8° C., segmentation was restricted to a few superficial cells. In these, the adjacent area was characterized by regularly spaced, small centrospheres with associated asters and surrounded by pigment granules. Many of these were not associated with chromosomes or nuclear material and have been interpreted as cytasters.

Yolk and Free Yolk-nuclei

In the uncleaved portions of the egg the yolk underwent regular changes which were probably degenerative in character. The yolk platelets disappeared locally and large vacuoles were formed as the yolk products went into solution. Progressive coalescence of vacuoles produced large fluid-filled spaces which were distributed irregularly through the non-cellular portion of the egg (Fig. 7).

The free nuclei, especially in the areas more distant from the cleaved portion of the animal pole, became progressively larger and more irregular. In the earlier cleavage stages they appeared to undergo normal mitosis but in the blastula and later stages the mitoses became progressively abnormal and large, irregularly-lobed nuclei appeared. These nuclear complexes were usually surrounded by dense aggregations of pigment granules. Some nuclei are vacuolated and stain very lightly while others are massive and stain densely. These giant nuclei, however, are not characteristic only of colchicine-treated embryos. They were also observed by Hertwig (1897) in the meroblastic segmentation of the frog's egg under the influence of centrifugal force.

Summary

The treatment of the developing frog's egg with colchicine (concentrations from 1 : 10,000 to 1 : 100,000) in amounts sufficient to retard mitosis or cause a temporary arrest of the process, results in a varying proportion of the eggs exhibiting a meroblastic type of cleavage. The degree of restriction of cleavage to the animal-pole region varies from the formation of a small cap of cells to a condition in which cleavage may include almost the entire egg. The level of development and completeness of the embryo are directly correlated with the extent of restriction of cleavage. Such animal-hemisphere embryos may not be capable of attaining even a modified blastula stage. In many blastulae

only the roof of the segmentation cavity is cellular. The process of gastrulation is always greatly disturbed and in extreme cases may be limited to a modified involution. Frequently, neural plate with beginning neural folds, notochord, dorsal mesoderm and entoderm are differentiated. Embryos rarely develop beyond this level.

In many embryos there are scattered areas in which the cells are large and possess correspondingly large nuclei. These are tentatively interpreted as the result of tetraploidy, a phenomenon commonly produced in plants by colchicine. The free yolk-nuclei are characteristically abnormal.

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EFFECTS OF 2, 4-DINITROPHENOL ON THE EARLY DEVELOPMENT OF THE TELEOST, ORYZIAS LATIPES

A. J. WATERMAN [1]

(From the Thompson Biology Laboratory, Williams College, Williamstown, Mass.)

The stimulating action of 2, 4-dinitrophenol on metabolism and oxygen consumption has been demonstrated for many organisms both embryonic and adult (Shoup and Kimler, 1934, luminous bacteria; Bodine and Boell, 1938, grasshopper embryos; Root and Etkin, 1937, toadfish; and others). It raises the metabolic rate of female rats (Halpern and Hendryson, 1935) but does not accelerate development in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938). In sea urchin eggs this and other nitro- and halophenols simultaneously increase respiration and block cell division (Krahl and Clowes, 1938). The physiological action is discussed by Bodine and Boell (1938).

In this investigation a study has been made of the effects produced by 2, 4-dinitrophenol on the development of the egg of the teleost, *Oryzias latipes,* (*a*) when continued exposure is begun at late cleavage to optic vesicle stages, (*b*) when the chorion is and is not pricked, and (*c*) when the egg is exposed to a lethal concentration for various periods of time.

This substance is highly toxic. In *Rana pipiens* (Dawson, 1938) it either retards, produces abnormalities or completely arrests development depending chiefly on the concentration. Among the abnormalities produced are: persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae. These results are somewhat comparable to what others have obtained in amphibia with high temperature, thyroxine, x-ray exposure, and dilute Ringer's solution. Buchanan (1938) found that such respiratory effects as this substance may have do not compensate for the retarding effect of low temperature on the development of *Amblystoma.* The effects on gastrulation are especially interesting because of the numerous studies of exogastrulation which have been made in both amphibia and echinoderms.

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Materials and Methods

Oryzias latipes (*Aplocheilus latipes*) is a small oviparous cyprinodont teleost, measuring about one inch in length, which is also known as the Japanese Medaka. This fish breeds well in the balanced aquarium and can stand low temperatures. Its small, colorless, transparent eggs are laid almost daily over long periods and remain attached to the cloacal region of the female until they are brushed off on the aquarium vegetation. The attachment occurs by the numerous processes peculiar to the egg chorion. Females lay from two or three to as many as twenty-five or more eggs at one time. If the eggs are regularly removed, they will continue to lay for several months. Otherwise the presence of the eggs appears to retard and later to inhibit egg production. Development is rapid and hatching occurs about fourteen to eighteen days after laying. Several factors may be responsible for this variation in hatching time, i.e. temperature possibly, inherent variation in developmental rate of embryos from the same and different females, and the time the eggs are laid in the reproductive period. At the end of the first day after laying, the eggs are in a late cleavage stage. The optic vesicle stage is reached during the second day and on the third to fourth days the heart is beating and differentiation is well advanced.

For the present study a 1 per cent stock solution of 2, 4-dinitrophenol was made according to the method of Halpern and Hendryson (1935), and sufficient amounts added to spring water to make the desired solutions. Concentrations of 1 : 10,000 up to 1 : 1,000,000 were used, but the most significant results appeared at concentrations from 1 : 40,000 up to 1 : 200,000. For the most part the embryos remained in the solutions for the entire experimental period. Eggs were also placed in a known lethal concentration for the late cleavage stage (1 : 10,000) and samples removed at intervals thereafter to fresh water. Since the egg is surrounded by a chorion, several studies were made in which the chorion was pricked. All experiments were made at room temperatures. Exposure was begun at four developmental stages; early and late cleavage, closure of the blastopore, and optic vesicle.

Experimental

In general the most conspicuous effect upon development is a retardation in both growth and differentiation which is proportional to the length of exposure and to the concentration of the drug, but there is variation in different individuals of a culture as some are more susceptible than others. No evidence of any stimulation was seen either in the developmental rate or in the hatching time nor were any cases

of exogastrulation obtained. The results obtained in one experiment are shown in Table I which is a typical example of the many that were

TABLE I

Effect of 2, 4-dinitrophenol on early development of *Oryzias latipes* (Late cleavage stage)

Experiment	Concentration	Age in days	Number dead	Average heart-beat (No. of secs. for 50 beats)	Heart-beat (low and high)	Temperature when examined	Comments	Hatching time
21	Control	7	None	28.7	24 34	25° C.		1 in 18 days 1 in 21 days 5 in 25 days
	1 : 40,000	7	None in 8	35.2	33 42	25° C.	Markedly retarded. Few have no circulation and rudimentary heart.	All dead 13 days after laying.
	1 : 80,000	7	1 in 14	31.2	27.5 34.5	25° C.	3 retarded, others like control.	2 dead in 22 days, all dead in 25 days, none hatched.
	1 : 120,000	7	None in 15	29	26 32	25° C.	6 retarded	2 in 19 days 2 in 21 days 1 in 22 days 2 in 25 days All died soon after hatching.
	1 : 160,000	7	None in 14	28.6	26.5 31	25° C.	No retardation	1 in 18 days 8 in 19 days 2 in 21 days 1 in 25 days 2 died
	1 : 200,000	7	1 in 7	28.4	27 30	25° C.	No retardation	1 in 18 days 5 in 25 days

tried. Concentrations greater than 1 : 10,000 inhibit all further development beyond possibly a few cleavages, while in a solution of 1 : 160,000 development is normal and the embryos hatch about the same time as the control. Although development occurs in concentrations greater than 1 : 90,000, it is very retarded, causing early deaths and no hatching. The effect of the drug is cumulative with time and retardation becomes increasingly evident with continued exposure to strong concentrations. These observations confirm those of others on the high degree of toxicity of this substance.

Except for the difference in the effective concentrations and the formation of certain abnormalities, the effects of 2, 4-dinitrophenol on the development of this fish closely duplicate those found by Dawson on the frog, and by Solberg (1938) for *Fundulus* embryos exposed to X-rays. The survival of the embryos is directly correlated with their

sensitivity to the drug. It is generally difficult to duplicate the results of one experiment with eggs taken from the same or different females on successive days. Another comparable observation is that temporary developmental inhibition does not necessarily cause irreversible injury, providing the exposure is not too long.

Both the controls and the experimental animals were run at the same temperature. This was always that of the laboratory and it varied between 24° and 27° C., with an average around 25°. This slight variation probably did not influence the action of the drug since Dawson found that in the frog the results were apparently not greatly modified by temperature. However, a large proportion of his animals showed arrested and abnormal development at 6° to 12° C. This is far below the temperature range of the present study. In *Bufo,* Buchanan found that the toxicity is higher at 21° than at 6° C.

The heart and extra embryonic vascular system are particularly sensitive. Since the rate of heart beat corresponds roughly to the concentration and length of exposure, it serves as a check on the other effects observed. However, no stimulation was observed. Concentrations greater than 1 : 130,000 progressively slow up the rate and amplitude of the heart beat and also provoke structural irregularities. Deformities of the heart lead to interrupted and poor circulation which in turn provokes other abnormalities as development progresses. These embryos die early. In concentrations greater than 1 : 40,000 the extra-embryonic circulation never becomes established and the heart is rudimentary although other embryonic differentiations may take place, i.e. nervous system, sense organs, body form, and sometimes somites. Blood corpuscles develop in all embryos but the quantity is less in the more retarded types. Blood vessels are also affected as, in the extra-embryonic area especially, some may be distended with blood while others remain unconnected.

Deformities in the myotomes and a reduction of their number are of common occurrence. The number may especially be reduced in the tail. Here the irregularities bring about shortening and curious shapes and positions of e tail. It often becomes bent at odd angles, twisted, curved or shortened. The tail fins may fail to develop but typical paired appendages are usually present in less abnormal cases. Embryos of these types did not hatch.

The nervous system and sense organs exhibit a variable response to the drug. In the stronger solutions some development stops at a neural tube stage while other embryos show the presence of the optic vesicle with but slight indications of the brain. In others the nerve cord is shortened or deformed. Often the neural canal is filled with a mass

of cells. Different regions of the brain are often missing, enlarged or abnormal on one side or the other. Very little effect is observed on the ears. The optic vesicles often fail to invaginate or show a variable amount of pigmentation. A size difference is also noticed as well as a malformation of the lens.

No particular type of teratological embryo is produced by dinitrophenol, but the production of uniform abnormalities is fairly consistent. Gastrulation either occurs normally, is retarded, or is entirely inhibited. If the exposure is not too long a few of the retarded embryos

TABLE II

Sensitivity of different developmental stages to a lethal solution of 2, 4-dinitrophenol (1:10,000)

Stage	Length of exposure before transfer to fresh water					Development during exposure
	4 hrs.	10 hrs.	21 hrs.	29 hrs.	45 hrs.	
Early cleavage	Retarded in size and development.	Small optic vesicle or rudimentary nerve tube. Some dead.	Dead			None
Late cleavage	Retarded in size and development.	Small optic vesicle stage.	Small optic vesicle stage.	Dead		None
Closure of blastopore	Slight retardation in some.	Smaller and thinner embryos. Slight pigmentation of eyes.	No development beyond optic vesicle or young non-pigmented optic cup stage. Rudimentary heart. No circulation.	No development beyond young optic vesicle stage.	Dead	None. Perhaps slight increase in cell number.
Optic vesicle	No effect		Slight retardation. Slower heart rate.		Marked retardation. No heart action. 96 hrs. exposure did not inhibit development.	Yes, but at a slower rate.

The above results are those seen after the embryos had been in fresh water for 3–4 days following exposure.

are able to complete gastrulation and to continue development in fresh water. Otherwise they give cases of persistent yolk plug which also occur in some instances during the experiment. Subsequent development is very atypical and death soon ensues. The inhibited blastulae do not increase in size and development goes no farther except for a few irregular cleavages especially noticeable at the periphery. None of these are able to gastrulate in fresh water. Unlike the frog, no living cases of exogastrulation were seen.

The results of the study of the relative sensitivity of several developmental stages to dinitrophenol are summarized in Table II. They show that the sensitivity decreases at least from the early cleavage stage to the optic vesicle stage and in the latter case development is able to continue, but more slowly, in a lethal solution which quickly kills the earliest stage tested.

Discussion

The resistance of fish embryos to the experimental conditions described in this study varies at different stages. At the optic vesicle stage they are much less sensitive to 2, 4-dinitrophenol than at the early cleavage stage. Solberg (1938) has likewise observed this to be true of *Fundulus* embryos exposed to X-rays. The earlier in development that the embryos are treated, the greater are the deformities. During the gastrula stages there is again a slight increase but thereafter the sensitivity declines rapidly and larger doses are required to modify development. Changes also occur in the sensitivity of different organs of the fish embryo to X-rays and dinitrophenol. As differentiation progresses the organs become more resistant; the time depends on the time of appearance and the rate of differentiation.

There was no special difference noted in the adjustment of early and late cleavage, or even older stages to the experimental solutions. Except for the question of sensitivity, early and late stages gave almost comparable effects. Dawson (1938) found this the case in his study of the effects of dinitrophenol on the development of *Rana pipiens* when treatment was begun at the 2-cell and early blastula stages. However, under the influence of high temperature, Hoadley (1938) found that the behavior of frog eggs is different immediately after the first cleavage from what it is at the 128-cell stage. There was a more complete adjustment to the new conditions on the part of the first group.

The suppression of cell division by dinitrophenol appears to be a common phenomenon. First reported (Krahl and Clowes, 1938) for sea urchin eggs, it has subsequently been found to occur in the frog (Dawson, 1938) and in the present study on the fish. At first this effect is hardly noticeable but with continued exposure mitosis is almost suppressed. The rate of this disturbance depends on the concentration of the drug. At one extreme development may be stopped at such an early stage as the optic vesicle, while at the other quite typical embryos may develop. However, the latter are noticeably smaller than the control, die sooner, and never hatch. Solberg (1938) finds that following X-ray exposure, the mitotic index of *Fundulus* embryos changes which accounts, at least in part, for the changes in sensitivity. The close

parallelism of the two changes during early development seems to him to be more than coincidental.

The chorionic membrane of the *Oryzias* egg is permeable to dinitrophenol but some difference is noted in the effective concentrations between cases of whole membranes and those in which the chorion is pricked. Even further differences might be noted if this membrane were entirely removed. The drug evidently penetrates slowly and higher concentrations and longer exposures are necessary for the intact membrane. This may account for the difference in the effective concentrations reported for the frog and *Bufo*.

In vertebrates, studies of exogastrulation have been limited thus far to the amphibia. The period of gastrulation is a critical one. Hoadley (1938) has shown that under the influence of supra-maximum temperatures early gastrulae and very late blastulae are unable to complete gastrulation and die. Early blastulae form giant blastulae which never undergo gastrulation. Many other environmental conditions are known to affect or modify amphibian gastrulation (cf. Dawson, 1938); dilute Ringers solution, X-ray exposure, thyroxin. Dawson obtained persistent blastulae, arrested early gastrulae, persistent yolk plugs and exogastrulae through the use of 2, 4-dinitrophenol. These results show that exogastrulation in amphibia is no more of a specific reaction to any particular agent than has been found to be the case among echinoderms (Child, 1936). They have all followed instances of developmental disturbance and arrest.

In the present study no cases of living exogastrulae were seen. Either gastrulation was inhibited entirely and the persistent blastulae remained as such until cytolysis and death occurred or gastrulation was retarded or stopped during the process. Cases of persistent yolk plug occurred occasionally. In all these interrupted types, the origin and the differentiation of the organ rudiments and even body formation were restricted. There was no indication of a differential effect on gastrulation alone. It would have been interesting to see what form exogastrulation would take in a telolecithal egg.

Certain types of abnormalities occurring in the embryos under the influence of 2, 4-dinitrophenol have been reproduced in fish by other agents and in the amphibia by various agents. For example, in *Fundulus heteroclitus* Solberg (1938) obtained comparable effects with X-rays on the development of the circulatory system, myotomes, nervous system, etc. Reference should be made to Dawson (1938) for work on the frog embryo. In general no special type of teratological embryo was produced but results were fairly consistent. As Dawson says, none of the effects obtained by treatment with dinitrophenol can be inter-

preted as being produced specifically by the drug but are such as might follow any treatment which disturbs the orderly processes of development and differentiation. If the increase in metabolic rate is one of the factors involved it is difficult to correlate this idea with the results secured by X-rays and by other agents tested on amphibia.

From the present study it would appear that 2, 4-dinitrophenol does not stimulate gastrulation or development in *Oryzias latipes* and, in the effective concentrations employed, it acts like any toxic agent. As such it retards, inhibits and disturbs normal orderly developmental processes in proportion to the concentration and length of exposure. Its effects are cumulative with time. Various concentrations which did not produce any observable defects caused no reduction in the hatching time. This confirms in the fish the observations of others that the drug does not accelerate development or metamorphosis in amphibian embryos (Cutting and Tainter, 1933; Dawson, 1938; Buchanan, 1938).

Dawson suggests that the increase in the metabolic rate may be one of the factors involved in the effects produced by 2, 4-dinitrophenol on the frog embryo since they resemble those caused by thyroxin (Baumann) and high temperature. According to Bodine and Boell (1938) the toxic effect may be due to the increased accumulation of toxic metabolic products of which the organism is unable to rid itself. In this connection it is interesting to note the conclusion of Lindahl (1936) from his study of the effect of SO_4-deficient sea water on the development of *Paracentrotus lividus* (the European sea urchin), since results were secured which in some cases are comparable to those of the frog and fish. SO_4-deficient sea water produces persistent blastulae, exogastrulae, ectoblastulae with cilia, etc. He considers that hydrocarbon metabolism dominates at the animal pole while protein metabolism dominates at the vegetal pole. The toxic action of this deficiency is caused by the accumulation of toxic phenolic derivatives coming from protein metabolism.

Summary

A study has been made of the effect of 2, 4-dinitrophenol on the development of four embryonic stages of the teleost, *Oryzias latipes,* i.e. early and late cleavage, closure of the blastopore and optic vesicle stages. Both the sensitivity of the whole organism and constituent parts to the drug decreases with age and differentiation. The general effect is cumulative and proportional to the concentration and length of exposure. In addition to inhibitory and retardative effects, other developmental abnormalities include those of the heart and blood vessels, myotomes, nervous system, body shape, etc.

Concentrations of 1:10,000 up to 1:1,900,000 were used, but the most significant results appeared at concentrations from 1:40,000 to 1:200,000. When the chorion is pricked there is some difference in the effective concentrations. No evidence of any stimulation was seen either in the developmental rate or in the hatching time. Gastrulation was either inhibited or retarded and no examples of exogastrulation were seen. If exposure has not been too long, recovery takes place to a more or less extent in fresh water.

Some of the results of the present study have been duplicated in the amphibia by various agents and in fish by X-rays. In general no special type of teratological embryo was produced but the results are fairly consistent and reproducible. It does not seem possible to attribute the effects to a specific action of this drug and hence to increased metabolic and respiratory rates alone, unless the change produces toxic products of which the embryo is unable to rid itself.

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EFFECT OF TEMPERATURE UPON SHELL MOVEMENTS OF CLAMS, VENUS MERCENARIA (L.) [1]

VICTOR L. LOOSANOFF

(From the U. S. Biological Laboratory, Milford, Connecticut)

Introduction

Knowledge of the effect of temperature on the physiological activities of mollusks is essential to the solution of many problems of shell-fisheries. Galtsoff (1928), in his studies of the effect of temperature on oysters, demonstrated its bearing on the problems of oyster culture and sanitary control of the oyster industry. His studies and those of Nelson (1921) and Hopkins (1931) added greatly to our knowledge of this phenomenon in relation to oysters, but little or nothing is known about other Pelecypoda. This study deals with the effect of temperature upon the shell movements of the hard-shell clam (*Venus mercenaria* L.).

In Pelecypoda, respiration and feeding are influenced by three factors, namely, the rate of activity of cilia of the gill epithelium, changes in size of the ostia, and by the movements of the shell-valves. It has already been shown (Galtsoff, 1928) that low temperatures induce hibernation in oysters. During the hibernation period the animal is unable to feed because of the disturbances of the gill mechanism, and because of closure of shells. It is of interest, therefore, to determine the temperature at which an animal enters into the hibernating stage and whether or not there is a definite correlation between the shell movements of a mollusk and the temperature of the surrounding water.

Experiments on the effect of temperature upon shell movements of the clam (*V. mercenaria*) were begun on November 17, 1934, and continued until July 29, 1935. They were conducted in a large outdoor concrete tank, 10 by 20 feet, at the laboratory of the U. S. Bureau of Fisheries in Milford, Conn. The tank was so constructed that the water entered it only at the last third of each flood tide and flowed out only partially at the ebb. Thus, the water in the tank was partly renewed twice every twenty-four hours. At low water stages the tank retained approximately 4,000 gallons of water. Because of this arrangement rapid changes in the temperature and salinity of water in the tank were rather uncommon. A roof over the tank protected it from the hot sun

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and heavy rainfalls, facilitating still further the maintenance of a comparatively even temperature and salinity.

Methods

Altogether 399 records from 47 different clams were obtained, each representing the movements of a clam's shell-valves during a 24-hour period. Usually the records of two clams were taken simultaneously. Clams were attached to a recording apparatus for varying periods of time ranging from one to eighteen days. During the course of this work the water temperature in the experimental tank varied from — 1.0°

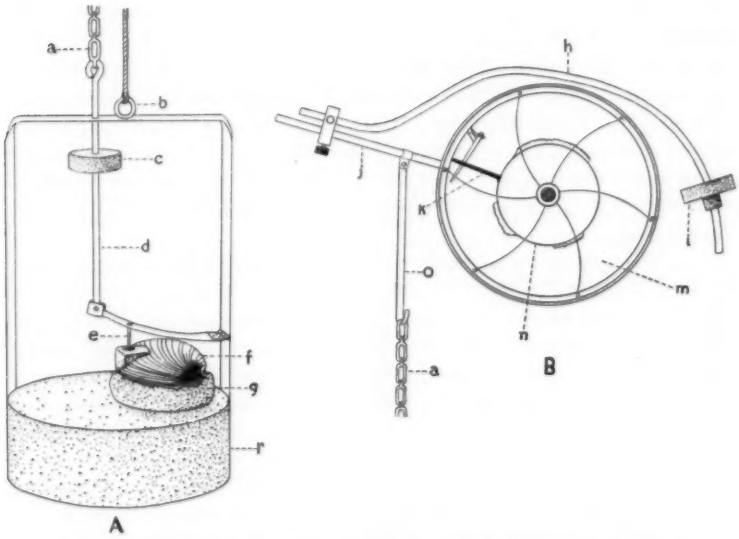

Fig. 1. Apparatus employed in studies of shell movements of clams. Description in text.

to 28.0° C., thus covering almost the entire range of temperature to which clams are subjected under natural conditions. The temperature of the water was recorded continuously by Brown's recording thermometer which was checked frequently against thermometers certified by the U. S. Bureau of Standards.

Each clam was immobilized by imbedding one of its shell-valves in a mixture of sand and cement. The animals were connected to the lever of a recording apparatus in such a way that each movement of the shell was recorded on a chart. The apparatus (Fig. 1) consisted of two main

parts, a foundation for keeping an experimental animal in a desired position (*A*), and a Foxboro recorder (*B*). An animal (*f*) imbedded in a block (*g*) of sand-cement mixture was placed on a large round concrete base (*r*) and connected to a recording instrument by a rod (*d*) and a chain (*a*) which was attached to a hook (*o*) of the recording apparatus. A flat piece of cement with a hole in the center was sealed to the free shell-valve of a clam, and on it rested a fine metal pin (*e*). By moving the nut (*i*) along the bent rod (*h*) the weight of a structure connecting the animal to the recording apparatus could be very accurately counterbalanced. This arrangement made it possible to eliminate any unnecessary pressure which would be exerted upon the clam. The rod (*j*) was connected to a writing pen (*k*) which recorded each movement of a clam shell upon the chart. By moving the hook (*o*) along the rod (*j*) the record of valve movement on a chart could be increased or decreased, as desired. In these experiments the distance between the experimental animal and the recorder was about 10 feet. The depth of the water over the clams varied from 4 to 7 feet depending upon the stage of tide.

While the time recorder has already been employed by other investigators in studying the shell movements of mollusks (Galtsoff, 1928), the method of holding the animal in a desired position and attaching it to the recorder, as described in this paper, is a new one.

The main advantage of using the apparatus described here, in preference to those employed by other investigators, is that it considerably simplifies the experimental work by rendering the handling and replacement of animals very easy. By pulling up the cord tied to the ring (*b*) of the foundation unit, the entire lower portion of the apparatus can be quickly raised from the bottom and the animal examined, or new animals substituted for the old ones. All manipulations in placing the animal in position and connecting it to the recording machine are performed out of water. The use of the chain (*a*) eliminates long rods, which were commonly used by other workers.

At each change of chart on the recording apparatus the experimental animal was tapped lightly to compel it to close its valves tightly. After this was achieved, a base line (*n*) was drawn on the chart by rotating it 360°. Having a distinct base line, every movement of a shell, no matter how slight, could be distinguished on the chart (*m*).

Results

Table I and Fig. 2 show the percentage of time the clams remained open at different temperatures. While subjected to very low temperatures (— 1.0° to + 1.9° C.) all the clams remained completely closed.

Altogether 56 records were obtained at such temperatures. At temperatures ranging from 2.0° to 2.9° C., with the exception of one case when an animal was open for about 5 hours, all the clams were inactive. A very slight increase in clam activities was noticed at temperatures ranging from 3.0° to 4.9° C. At the latter temperature the majority of experimental animals remained completely closed, but a few opened on several occasions, bringing the average of open time to 9 per cent. A sudden increase in clam activities was noted at 5.0° to 5.9° C., when the percentage of time open increased to 35 per cent. Such a pronounced increase in shell activities indicates that for many clams the critical hibernating temperature lies somewhere between 5.0° and 6.0° C.

TABLE I

Number of 24-hour records of shell activities obtained at each temperature ranging from −1.0° to 28.0° C., per cent of total time, and average time shells remained open at each temperature during 24-hour period.

Temperature	No. of records	Percentage of total time shells opened	Average time shells opened during 24 hours		Temperature	No. of records	Percentage of total time shells opened	Average time shells opened during 24 hours	
°C.			*hours*	*minutes*	°C.			*hours*	*minutes*
−1.0– 0.1..	18	—	—		14.0–14.9	8	88	21	7
0.0– 0.9..	8	—	—		15.0–15.9	16	81	19	26
1.0– 1.9..	30	—	—		16.0–16.9	6	78	18	43
2.0– 2.9..	26	1	0	14	17.0–17.9	12	84	20	10
3.0– 3.9..	30	4	0	58	18.0–18.9	6	89	21	22
4.0– 4.9..	32	9	2	10	19.0–19.9	4	76	18	14
5.0– 5.9..	22	35	8	24	20.0–20.9	12	86	20	38
6.0– 6.9..	6	29	6	58	21.0–21.9	6	90	21	36
7.0– 7.9..	12	65	15	36	22.0–22.9	10	86	20	38
8.0– 8.9..	22	67	16	5	23.0–23.9	4	78	18	43
9.0– 9.9..	22	74	17	46	24.0–24.9	16	69	16	34
10.0–10.9..	4	88	21	7	25.0–25.9	9	71	17	2
11.0–11.9..	8	85	20	24	26.0–26.9	6	83	19	55
12.0–12.9..	26	84	20	10	27.0–27.9	4	89	21	22
13.0–13.9..	14	71	17	2					
					Total	399			

At 7.0° to 7.9° C. all the clams were open some of the time, bringing the average time open to 65 per cent. From 8.0° to 10.9° C. a further increase in shell activities was shown. At 10.9° C. the animals remained open 88 per cent of the total time. As the water warmed from 11.0° to 27.9° C. no definite correlation between the temperature of the water and the length of time the shells remained open could be detected. Between these two points, the percentage of open time fluctuated between 69 and 90.

The individual differences displayed by the clams used in these experiments were quite significant. For example, while some of the animals exposed to temperatures of 5.0° to 5.9° C. remained open for the entire period of twenty-four hours, others did not open at all.

At low temperatures the clams may remain completely closed for several days. For example, one clam had its shell closed from noon on December 16, 1934, until noon on January 3, 1935, a period of 18 days. The temperature of the surrounding water during that period fluctuated from — 1.0° to 6.5° C. Another clam remained completely

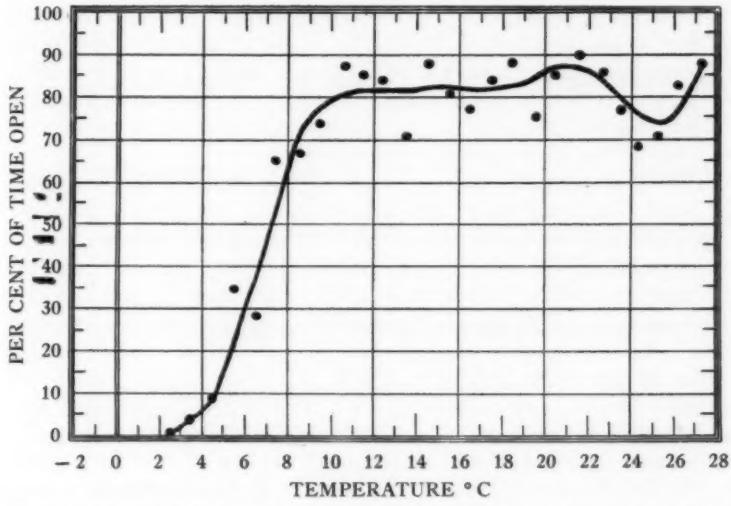

FIG. 2. Percentage of time clams remained open at different temperatures during 24-hour periods.

closed from March 8 to March 19, 1935. In several other instances the animals' shells remained closed for various periods extending from 3 to 6 days. In every one of these cases, the record of shell activity showed no deviation from the base line, thus indicating that not even a slight movement of the shells took place during such a prolonged period. Somewhat similar observations were made by Galtsoff (1928) on one specimen of *O. virginica* which remained tightly closed for 67 hours when left in cold water of a temperature varying from 0.5° to 1.6° C.

Galtsoff (1928), in his work on *O. virginica,* could not find a definite correlation between the effect of temperature and the time the shells of

oysters remained open. This was due, probably, to the fact that in his experiments the temperature of the water varied within the comparatively narrow range of 13.0° to 22.0° C. No systematic observations were performed at low temperatures. Similar conclusions could have been reached in this work if the observations had been confined to higher temperatures only. As has been mentioned already, there was no correlation found between the time the clam shells were open and the temperature increase from 11.0° to 27.9° C. If, however, the shell movements are examined at temperatures ranging from 0.0° to 11.0° C., the correlation between the rise in temperature and the gradual increase in duration of openness of the valves is noticed. This is especially evident within the range of 3.9° to 10.9° C., in which the average period of openness increases from 4 to 88 per cent (Fig. 2). Such an increase indicates that as soon as the water temperature gradually rises from the hibernation point to 11.0° C., the factors or conditions controlling the opening of the shells approach the optimum.

The present study, however, does not provide definite information as to the exact temperature at which the optimum conditions for opening of clam shells are reached. Within a 11.0° to 27.9° C. temperature range the shells are open from 69 to 90 per cent of the total time, but the time the shells of the animals remain open does not increase proportionally to the increase in temperature. The highest percentage of time open is recorded at temperatures 21.0° to 22.0° C. when the clams remained open 90 per cent of total time, or 21 hours and 36 minutes per 24-hour period. The average period of time the shells remained open at temperatures from 11.0° to 27.9° C. is 19 hours and 35 minutes. This figure closely resembles those obtained by some other investigators in their studies of oysters. Nelson (1921), basing his conclusions on the records of 3 oysters (*O. virginica*) kept under observation for 21 days, states that the animals remained open an average of 20 hours per day. Galtsoff (1928), from more numerous observations, concluded that the average period of time the shells of oysters remained open is 17 hours and 7 minutes per day. Hopkins (1931), working on *O. lurida,* found that the oysters were open and presumably feeding over 20 hours per day. From the study of the movements of clam shells and from observations of other investigators on oyster shell movements, it appears that under favorable conditions these pelecypods keep their shells open as long as possible.

Experimenting with *O. lurida,* Hopkins (1931) came to the conclusion that it is not so much the existing temperature of the water which determines how long the shells remain open as it is the changes in temperature which occur. This sensitivity of oysters to temperature

changes varies in an inverse manner with the temperature of the water. Thus, a small drop in temperature causes closure of the shell if the temperature is well below the optimum, but produces no effect if near the optimum. In clams no such effect of sudden changes in temperature could be detected. For instance, on December 2, 1934, between 2:00 and 3:00 P.M., the water temperature in the experimental tank rose

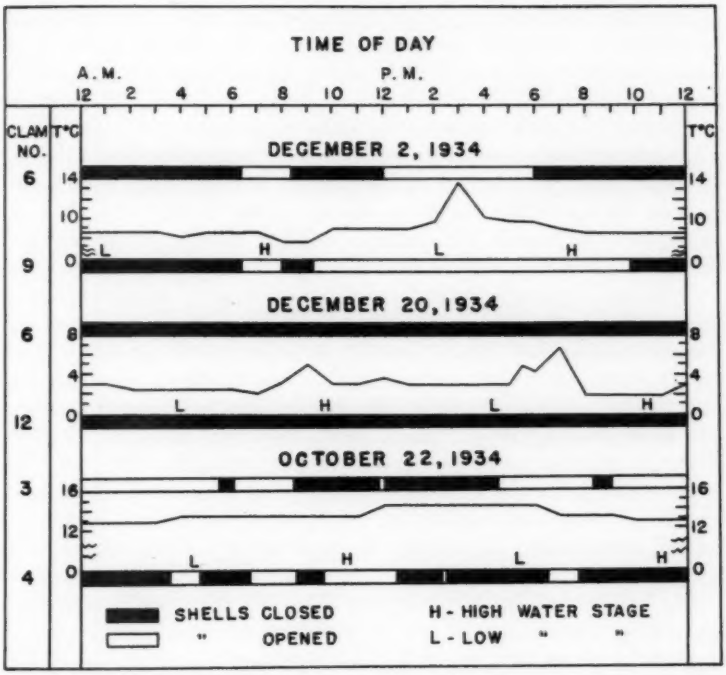

FIG. 3. Records of shell activities of clams and temperature of surrounding water during 24-hour periods. A sudden change in temperature (December 2 and 20, 1934) failed to cause the specimens to exhibit rapid closing or opening of shells. Shells closed and opened when temperature remained unchanged (October 22, 1934). No correlation between the tidal stages and shell movements could be observed. *H*—high-water stage, *L*—low-water stage.

from 9.5° to 13.5° C., and two hours later decreased to 9.5° C. (Fig. 3). Both of the experimental animals, whose shells were open, remained apparently undisturbed. In another case, on December 20, 1934, the water temperature in the experimental tank increased from 3.0° to 6.5° C. within two hours and then quickly dropped to 2.0° C. Neither of the two experimental animals showed any noticeable activities.

It appears that in *Venus mercenaria* the mechanism controlling the opening and closing of the shells is less sensitive to minor temperature changes than that of the oyster. In clams, the closing or opening of the shells is very often performed when the temperature of the surrounding water remains at the same point for some time. On the other hand, the closing or opening often occurred at decreasing temperatures, while again, in numerous other instances, these phenomena took place at increasing temperatures (Fig. 3, October 22, 1934). As far as can be judged by examining hundreds of the records obtained in this work, small changes in the temperature of the surrounding water do not directly influence the shell movements of clams.

During periods of openness the adductor muscles of the clam do not remain in the same position but relax and contract, indicating the changes in tonus level. Contraction of the muscles, although quite pronounced, seldom results in the complete closing of the shells. The data obtained help to answer the question whether there is a definite periodicity in contraction and relaxation of clam muscles, and, if so, whether such periodicity is affected by exposing the animal to different temperatures. Figure 4 represents the records of the shell movements of two experimental animals, Nos. 14 and 16, exposed to different temperatures ranging from about 0.0° to about 20.0° C. and having intervals of about 4.0°. The temperature of the water during these experiments fluctuated not more than $\pm$ 1.0° C. Each record represents the activities of the clam for a period of 12 hours, from 6:00 P.M. until 6:00 A.M. The night period is chosen in preference to daytime because the changing of charts in the recording apparatus, usually made in daytime, slightly disturbed the animals. By taking the night half of a daily record, more reliable information is available. The muscles controlling the shells of clams relaxed and contracted, sometimes at very brief, and at other times at prolonged intervals (Fig. 4). It is difficult, however, to find a definite periodicity throughout any of the 12-hour periods shown in Fig. 4. Although during several brief intervals the movements of the shells were of a definitely periodic type and exhibited a rhythmic character, more often they occurred as unsystematic and inconsistent. In this respect the shell activities resemble those of oysters, in which, according to Hopkins (1936), partial closures of the shells are sometimes periodic, sometimes only occasional and unorganized.

Clams whose shell activities are graphically shown in Fig. 4 present an opportunity of comparing the behavior of two individuals subjected to identical environmental conditions. With the exception of the records obtained at the temperature of 16.0° C., all other records of the shell activities of these two clams were obtained simultaneously. At the tem-

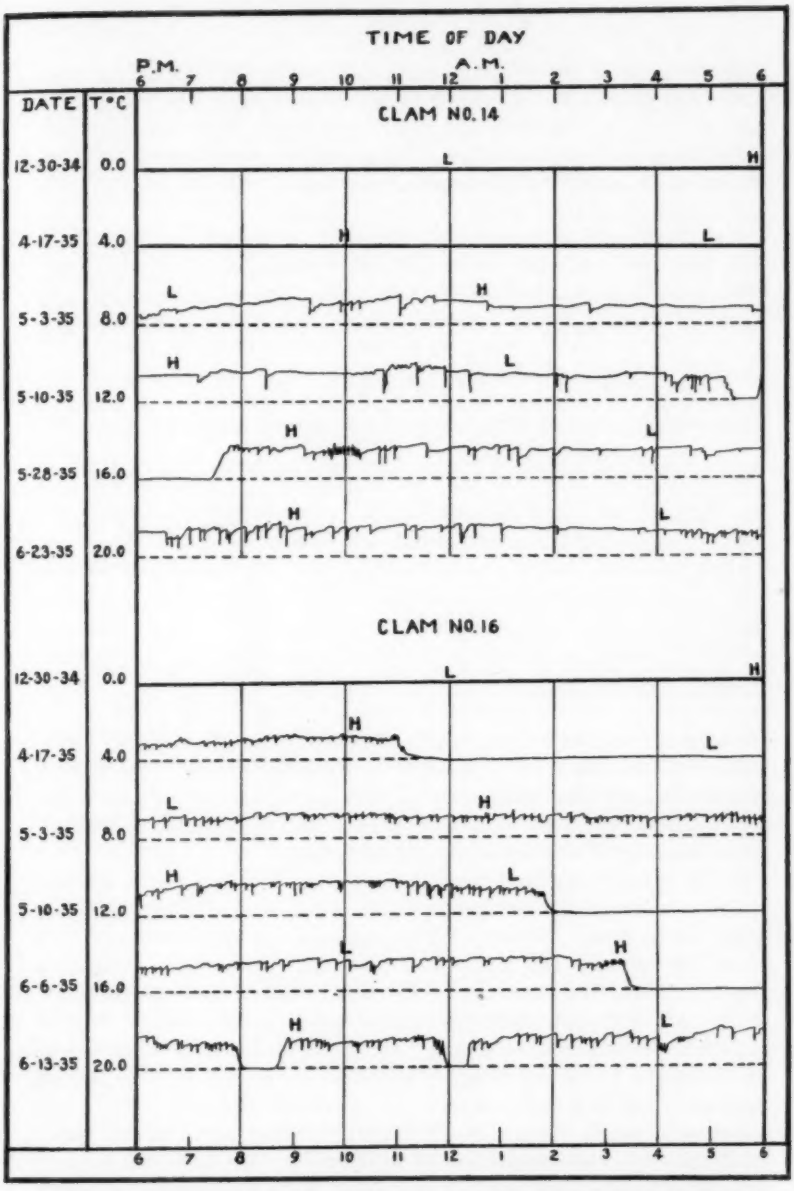

FIG. 4. Records of shell movements of two clams Nos. 14 and 16, exposed for 12-hour periods to different temperatures ranging from 0.0° to 20.0° C. Base line is indicated by series of dots. *H*—high-water stage, *L*—low-water stage.

perature of 0.0° C. both animals remained closed, thus exhibiting uniform behavior. At 4.0° C. one clam remained closed whereas the other was open part of the time. At higher temperatures both clams were active. The records show that there were considerable individual differences in the behavior of these animals although they were subjected to the same external environmental conditions. Such variability may probably be explained as due to internal causes.

In these experiments no definite correlation between the stage of the tide and shell movements of the experimntal animals could be detected. This is demonstrated in Figs. 3 and 4 which show activities of clams at different tidal stages. The failure of the experimental animals to react

TABLE II

Percentage of time the shells of clams remained closed at daytime and night when exposed to different temperatures.

Temperature classes	Number of records	Number of clams	Average percentage of daylight closed	Average percentage of night closed
0.0– 4.9° C.....	7	3	67.0	74.0
5.0– 9.9° C.....	24	6	36.0	8.0
10.0–14.9° C.....	27	5	20.0	9.0
15.0–19.9° C.....	40	6	18.0	23.0
20.0–24.9° C.....	46	9	23.0	18.0
25.0–28.0° C.....	19	5	23.0	20.0

in a definite way to tidal changes is attributed to the fact that the tanks, where the animals were kept, are so constructed that they are filled at flood tide only and retain part of this water during the ebb stage. Therefore, fluctuations in temperature and salinity of water in the tank were not as great as those in the adjoining harbor.

The material on hand permits the answer to the question whether there is a correlation between opening and closing of clam shells and light and darkness. Nelson (1921) suggested that such correlation does exist for oysters. His conclusions were based upon oysters lying on a natural oyster reef subjected to the usual changes in temperature, salinity, pH and other factors found in open coastal waters. Galtsoff (1928), working in the laboratory under the fairly stable conditions of a relatively constant water supply and lesser differences between diurnal and nocturnal illumination, found no correlation between periods of closure and daylight hours. Webb (1930), working on *O. edulis,* found that the onset and termination of daylight have but little influence on the behavior of oysters as judged by their valve movements. Hopkins

(1931) observed the diurnal variation in the amount of time oysters (*O. lurida*) remained open, but this, according to his opinion, could be directly correlated with temperature fluctuation. In the present study 163 complete (24-hour period) records were examined. All other records showing that the animal was either open or closed for the entire period of 24 hours were not taken into consideration. The period of daylight was taken as the time between sunrise and sunset. After averaging all the data it was found that the clams kept their shells closed during 25 per cent of the total daylight, and 19 per cent of the total time of darkness. Thus, it appears that whereas there was no diurnal variation in shell activities of the clams, the animals were closed for a somewhat longer period in daylight than in darkness.

Records, mentioned above, were obtained between April 10 and July 29, 1935, thus covering the period of 110 days during which the environmental conditions of clam existence were gradually changing. In that span of time the temperature of the water increased from 3.7° to 27.5° C. Such a change in temperature, from below hibernation point for the majority of the clams to the maximum temperature of the year, offered an opportunity to determine whether the ratio between time of shell closure at daylight and at night varied with the rise in temperature as the season progressed. To answer this question all 163 records were arranged in six temperature classes, 5.0° apart. In referring to Table II, which shows the results obtained, it should be remembered that because the number of records is different for each temperature-class the direct comparison between classes is somewhat difficult. However, by studying this table it will be noted that the ratio of time the shells remained closed in daylight or at night varied considerably with the temperature. Such variation is especially significant at the temperature ranging from 5.0° to 14.9° C. At these temperatures the animals had their shells closed for a much longer time in daylight than at night. At present no explanation can be advanced for the occurrence of this phenomenon.

Summary

1. A new apparatus, by means of which the shell activities of many bivalve mollusks can be measured and recorded, is described.

2. The analysis of 399 daily records of shell activities of 47 clams, subjected to temperatures ranging from — 1.0° to 28.0° C., showed that the length of time which the animals remain open partly depends upon the temperature of the surrounding water.

3. For the majority of clams hibernation begins soon after the water temperature decreases to 5.0° and 6.0° C. At lower temperatures the

clams may remain completely closed for very long periods. No shell movements were exhibited, and no disposal of ejecta occurred.

4. Within the temperature range of 3.9° to 10.9° C., the average period of openness increased from 4 to 88 per cent of the total time, showing a correlation with the rise of temperature.

5. There was no correlation between the duration of openness of the clam shells and the temperature increase from 11.0° to 27.9° C. Within this temperature range the shells were open from 69 to 90 per cent of the total time, but the percentage did not increase simultaneously with the increase of water temperature.

6. The highest percentage of time open was recorded at temperatures 21.0° to 22.0° C., when the clams remained open 90 per cent of the total time, or 21 hours and 36 minutes per 24-hour period.

7. Small changes in the temperature of the surrounding water did not influence the shell movements of clams.

8. There appeared to be no definite periodicity in the clam shell movements. During brief intervals, the shell movements may be of a periodic type and exhibit a rhythmic character, but generally they appeared to be unsystematic and inconsistent.

9. There were considerable individual variations in the behavior of clams kept under identical environmental conditions.

10. Under the conditions of the experiments no definite correlation between the stages of tide and the shell movements of the animals could be detected.

11. The animals were closed for somewhat longer periods in daytime than in darkness.

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THE ACTION OF CERTAIN DRUGS ON THE INSECT CENTRAL NERVOUS SYSTEM

K. D. ROEDER

TUFTS COLLEGE, MASSACHUSETTS

The purpose of this paper is to describe the effects of certain drugs on general reflex activity in insects, and to compare the pharmacology and physiology of the insect and vertebrate nervous systems. The literature reveals few positive observations of the effect of injected drugs on insects, though in general it seems that insects are less responsive than other invertebrates.

MATERIAL AND METHODS

The material for these experiments consisted of mature female praying mantids (*Mantis religiosa*) and mature cockroaches (*Periplaneta americana*). The various drugs used were made up in .6 per cent saline shortly before injection. The drugs were introduced by injection into the head capsule with a small syringe and fine hypodermic needle. Since the insect circulation is rather sluggish, there is only slight general distribution of an injected solution, and the site of injection determines to some extent the effect of a drug. An injection of .01 cc. into the head capsule appears to reach the supraesophageal and to some extent the subesophageal ganglion, but has little or no effect on the rest of the nerve cord. Records were kept in the form of written observations and 16 mm. moving pictures. Little attempt will be made to present quantitative results, since it is difficult to inject an exactly measured dose into an unanaesthetised insect, and there is no satisfactory way of quantifying the resultant change in behavior without giving false impressions.

EXPERIMENTAL

Strychnine

The normal posture and degree of activity of the mantis are variable, and have been described at length (Roeder, 1937). Injection of .02 mg. strychnine causes a slight drop in tonus and decrease in antennal vibration. This effect is very transitory, lasting only a few minutes. Larger doses of from .05 to .1 mg. result in a pronounced departure from the normal within 3 to 5 minutes. First the antennal vibrations become

slower and the movements of the mouth-parts very sluggish. With large doses movements of the head appendages cease entirely. The tonus of the trunk muscles drops, and the insect takes on a crouching posture owing to the sagging of the long prothorax. The degree of locomotor activity varies, though at first there is an increase, the insect walking slowly and restlessly. The movements of the legs are coördinated, and identical with normal locomotor movements. Owing to insensitivity of the head appendages and the crouching posture, the mantis is unable to climb up or over any obstacle, and since it moves in a straight line, it eventually becomes wedged in a corner or under some object. Mechanical stimulation of the antennae or photic stimulation of the eyes fails to produce any response, and the whole head appears to be quite insensitive. These symptoms: a drop in tonus, increased locomotor activity, and paralysis of the head appendages also follow destruction of the cerebral ganglia, therefore the effect of strychnine on the mantis central nervous system is entirely depressant. Experiments with cockroaches yielded essentially similar results. The insects became very quiet, and there was little mouth-part or antennal movement. Both mantids and cockroaches recovered completely from the effects of strychnine within an hour.

The effect of strychnine on these insects is then in direct contrast to its well-known effect on the vertebrate spinal cord, where it supposedly lowers thresholds between neurons in the cord, and is capable of turning an inhibitory process into an excitatory one. Many invertebrates show a comparable reversal of inhibition (Knowlton and Moore, 1917; Moore, 1918; Crozier and Federighi, 1924; and Crozier, 1927, 1930), though strychnine is recorded as having little or no effect on insects (Crozier, 1922; Crozier and Pilz, 1924). Many other papers are summarised in a review by Poulsson (1920). The only vertebrate organ which is depressed by strychnine is the heart (Poulsson, 1920).

Pilocarpin

This drug belongs to a group which has a specific stimulating effect on parasympathetic effectors (Kuntz, 1934). In vertebrates it increases salivary secretion, produces vasodilation, has a vagus-like effect on the heart, and increases contractility in the stomach and intestine, while its action is blocked by atropin (Dixon and Ransom, 1924). No record of its effect on the vertebrate central nervous system could be discovered.

If pilocarpin is injected into the head capsule of a praying mantis, it produces a state of great excitation. The minimum dose producing observable effect is about .01 mg. while doses of from .02 to .1 mg. bring about a change in behavior within thirty seconds. The walking legs become flexed, the prothorax raised, and the head ventrally flexed.

apparently due to an increase in tonus of the ventral neck muscles. There is an increase in amplitude and rate of antennal movement, and the mandibles are opened and shut, producing a clicking sound. There are spasmodic movements of the raptorial legs, consisting of flexions and extensions, and occasionally the whole body is swayed rapidly from side to side. All reflex activity increases except locomotion. Only three out of thirty insects showed any tendency to walk, either spontaneously or on stimulation. Recovery occurs rapidly and the insect is normal within an hour.

High muscle tone and inhibition of locomotor activity are typical of an insect with the right and left cerebral ganglia separated. This is interpreted as being due to increased activity of the tonus and inhibitory locomotor centres when released from the inhibitory effect of the contralateral ganglion (Roeder, 1937). Since pilocarpin when applied to the cerebral ganglia of an intact insect has the same effect as median division, it must have an excitatory action upon these centres. This can be substantiated by removing the cerebral ganglia prior to injection. An insect so treated walks rapidly in a crouching position. Since the cerebral centres are now absent the pilocarpin exerts its excitatory effect upon the locomotor centre in the subesophageal ganglion with consequent activity.

Pilocarpin has a comparable effect on cockroaches. A solution of the drug was injected into the head in doses of approximately .01 cc. A solution of 1 : 500 produced immobility and apparent paralysis but solutions of 1 : 500 to 1 : 5,000 caused intense excitation. It is impossible to give an adequate description of the behavior because so many things occur simultaneously. The mouth-parts and antennae are in continual movement, the head is moved from side to side and ventrally flexed, and cleaning movements are made with the legs, which are often abnormally extended. Occasionally there are violent cramps and spasms involving the whole of the body musculature and very rapid side-to-side swaying movements similar to those observed in treated mantids. More dilute solutions of 1 : 5,000 to 1 : 25,000 either have no noticeable effect or produce only a restlessness and general increase in activity. The above symptoms appear within one minute of injection and last for about thirty minutes. Recovery is usually complete. The excitation produced by pilocarpin seems to indicate a similarity between the nervous systems of these two insects and the vertebrate parasympathetic. This similarity leads to an investigation of the response to acetylcholine and eserine.

Acetylcholine and Eserine

Recent work has shown that acetylcholine acts as mediator between parasympathetic nerves and autonomic effectors, and between pre- and

postganglionic fibres in the autonomic ganglia. Naturally produced acetylcholine is destroyed very rapidly by the enzyme esterase, but it can be protected from destruction by a drug, eserine. By its protective action eserine renders autonomic effectors and postganglionic fibres more sensitive to introduced acetylcholine, and, by preventing the immediate destruction of naturally produced acetylcholine, can prolong the postganglionic response to a preganglionic stimulus (Cannon and Rosenblueth, 1937).

For these experiments cockroaches only were used, as mantids were not available. Acetylcholine hydrochloride was dissolved in .6 per cent saline, and injected into the head, in .01 cc. amounts. Strengths from 1 : 500 to 1 : 25,000 were used but in no case was any change in behavior noted. Since the acetylcholine was unprotected by eserine its effect may have been only transitory, and therefore would have escaped attention. In order to prolong the effect, if any, cockroaches were first injected with eserine. This drug had an unexpected effect, which was comparable with, but more pronounced than, that of pilocarpin. Eserine alone in dilutions of 1 : 250 to 1 : 500 produced an almost instantaneous spasm and immobility from which the insect did not recover, while solutions of 1 : 500 to 1 : 2,500 caused enormous reflex activity, which consisted of rapid antennal quivering, continuous mouth-part activity, movements of the head, great increase in extensor leg tonus and general spasms. Usually the insects did not walk, but one or two were so active that no observation could be made. Solutions more dilute than 1 : 5,000 either had no effect or produced only a slight restlessness.

It can be seen that this strong eserine effect made it impossible to test the effect of acetylcholine protected by eserine. Though the presence of acetylcholine in the nervous system of these insects has not been demonstrated, the action of eserine could be explained by its presence.

Atropin

The specific action of this drug is to block transmission of impulses in autonomic ganglia and to parasympathetic effectors. It also renders these structures insensitive to parasympatheomimetic substances such as acetylcholine and pilocarpin (Cannon and Rosenblueth, 1937). From the foregoing experiments it might be expected that atropinised insects would fail to respond to pilocarpin, and would also show a decreased reflex activity. The answer to the second question is somewhat doubtful. Atropinised cockroaches remain very inactive for long periods, though they are able to respond if disturbed. Cockroaches were first brought into a condition of great activity with .01 cc. of 1 : 500 pilocarpin, and

then subjected to an injection of atropin. This failed to decrease the pilocarpin effect to a noticeable extent. If the insects were first injected with atropin and then given an injection of 1:500 pilocarpin, they showed either normal or decreased activity in eight out of ten cases. Two insects showed a very brief phase of excitation, lasting a few seconds, and then became inactive, but in no case was there the prolonged and intense reflex activity which occurs if such a dose of pilocarpin is administered alone. Therefore, previous treatment with atropin protects cockroaches against the pilocarpin effect, just as it does in the vertebrate parasympathetic.

Conclusions

The following conclusions seem justified:

(1) The effect of strychnine on the reflex activity of the mantis and cockroach indicates that the central nervous systems of these insects and vertebrates differ in certain respects.

(2) The action of pilocarpin, eserine, and atropin on the reflex activity of these insects indicates that there is a pharmacological similarity between their nervous systems and the vertebrate parasympathetic.

It must be recognized that the experiments outlined above are suggestive rather than conclusive, since the methods used are in many ways unsatisfactory. First, there is no way of knowing the exact concentration of drug actually reaching the cerebral ganglia, partly because it is not an easy matter to inject small exact amounts into a struggling insect, and partly because of the sluggish and casual nature of the insect circulation. Second, there is no way of telling exactly what structures are being reached by the injected drug. From information available on the functions and interrelations of the cerebral ganglia in the mantis (Roeder, 1937) it seems highly probable that these structures are being directly affected. The great activity of the mouth-parts indicates that the subesophageal ganglion is also affected to some extent, though it is hard to say to what extent the drugs are affecting sense organs and neuro-muscular junctions. Third, observation of changes in the general reflex activity is a complex and unsatisfactory criterion. However, a satisfactory technique avoiding some of these difficulties has been developed, and it is hoped that more extended conclusions can be submitted in a later paper.

The similarity of the insect central nervous system and the vertebrate parasympathetic immediately suggests the presence of a chemical substance such as acetylcholine, which would serve as mediator in the transfer of impulses from neuron to neuron in the central nervous system. No proof of the presence of such a substance is presented here,

though the action of eserine is suggestive. Acetylcholine unprotected by eserine produces no noticeable effect when injected, but an insect is capable of such rapid and brief responses that one would expect very rapid destruction or removal of any substance mediating the transfer of impulses.

The question as to whether these findings apply generally to insects cannot be given an adequate answer. It has been reported that strychnine produces little or no excitation in caterpillars (Crozier and Pilz, 1924), while pilocarpin raises sensory thresholds in the same insects (Crozier, 1922), while crustacea (Clarke and Wolf, 1932; Viehoever and Cohen, 1937) and many invertebrates respond to strychnine in a vertebrate-like manner. It would seem that a generalization with respect to insects must await a further investigation.

Summary

1. Adult praying mantids and cockroaches were injected in the head with solutions of various drugs, and the changes in general reflex activity were recorded.

2. Strychnine caused a decrease in reflex activity in both insects, large doses causing complete cessation of antennal and mouth-part movement. This is contrary to its effect on the vertebrate spinal cord.

3. Pilocarpin and eserine bring about an increase in mouth-part and antennal movement, head movement and extensor leg tonus, and produce spasmodic twitching and general contraction of the body musculature. In the case of pilocarpin this effect is prevented by atropin.

4. Acetylcholine alone has no detectable effect on activity. Its effect, if any, when injected with eserine is masked by the excitatory effect of eserine.

5. It is concluded that, in their reactions to drugs, the nervous systems of the two insects studied show little similarity to the vertebrate central nervous system, but considerable similarity to the vertebrate parasympathetic.

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SOME PROPERTIES OF SPERM EXTRACTS AND THEIR RELATIONSHIP TO THE FERTILIZATION REACTION IN ARBACIA PUNCTULATA[1]

JOHN A. FRANK

(From the Marine Biological Laboratory, Woods Hole)

Since the early work of Piéri (1899), numerous attempts have been made to isolate substances from sperm which could be shown to function in normal fertilization. Extracts and filtrates of sperm, prepared by a wide variety of methods, have consistently proved inactive as parthenogenetic agents. The literature of this subject has been reviewed by Sampson (1926), who points out that any positive results reported may be ruled out on the basis of technical or interpretive errors. Largely because of these discouraging results, the search for the hypothetical "fertilizing substance" of sperm has been abandoned during the past decade.

The purpose of this investigation was to study certain properties of sperm extracts obtained by heating and filtering sperm suspensions of *Arbacia*. The experiments deal with (1) the agglutinating action of sperm extracts on *Arbacia* eggs, (2) the relation of such extracts to egg-water ("fertilizin" of Lillie, 1913, etc.), (3) the effects of sperm extracts on the fertilizing power of sperm, the fertilizability of eggs, and the development of fertilized eggs, and (4) the action of sperm extracts as parthenogenetic agents.

Materials and Methods

The experiments were carried out during the summers of 1934, 1937, and 1938 on the gametes of the sea-urchin, *Arbacia punctulata*. Eggs and sperm were obtained by the method described by Just (1928). After cutting out the lantern and peristome with scissors, the coelomic fluid was washed away with sea water and the urchin placed aboral side down over a Stender dish and allowed to shed. Mixed "dry" sperm from several males was kept in a test tube until required and mixed shed eggs were washed and placed in a small volume of sea water. Where large amounts of material were needed or when the

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animals shed poorly, the ripe gonads were removed with forceps, washed, and strained through cheesecloth. All instruments and glassware were rinsed thoroughly in tap and sea water before use to prevent chance contamination of eggs with sperm.

In preparing jellyless eggs, shed ("normal") eggs were treated with a mixture of 1.4 cc. N/10 HCl in 50 cc. sea water, washed, and examined in Chinese ink to insure the absence of jelly (Lillie, 1915*a*). This procedure removes the jelly from 90–100 per cent of the eggs without harming them.

Sperm extracts were prepared as follows: Sperm suspensions, made up in 10-cc. portions by adding a measured volume of dry sperm to sea water, were heated one minute over a small Bunsen flame. The albuminous sperm coagulum was removed by filtration through No. 5 Whatman filter paper. The filtrate, or sperm extract, was considered to be equal in concentration to that of the sperm suspension from which it was obtained. Thus a 10 per cent sperm extract was prepared by heating and filtering 1 cc. sperm in 9 cc. sea water.

In preparing and determining the fertilizin concentration of egg-water, Lillie's method (1914) was followed with slight modifications. Fresh unfertilized *Arbacia* eggs, which had stood at least fifteen minutes in a small volume of sea water, were lightly centrifuged and the supernatant fluid (egg-water) removed with a pipette. The presence of fertilizin was detected by adding a drop of egg-water to a drop of fresh 1 per cent sperm suspension and observing reversible agglutination of the sperm under the microscope. By making successive half-dilutions of the egg-water with sea water, a dilution is reached at which the sperm agglutinate for three to six seconds but above which no agglutination occurs. This dilution, by definition, contains one fertilizin unit and the fertilizin concentration of the original egg-water is thereby determined. For example, if an egg-water agglutinates sperm at 1/1600 dilution but not at 1/3200, the undiluted egg-water contains 1600 fertilizin units.

The Egg-Agglutination Reaction

When a few drops of *Arbacia* eggs are added to a small quantity of sperm extract in a tube, a striking reaction occurs. As each drop falls it sets into a dense red mass which remains suspended from the surface of the solution by a thin ribbon of coagulated eggs (Fig. 1, *A*). When the tube is shaken the ribbons coalesce to form a loose clot of eggs which usually floats at the surface of the extract (Fig. 1, *B*). A similar quantity of eggs, shaken in heated water as a control, forms

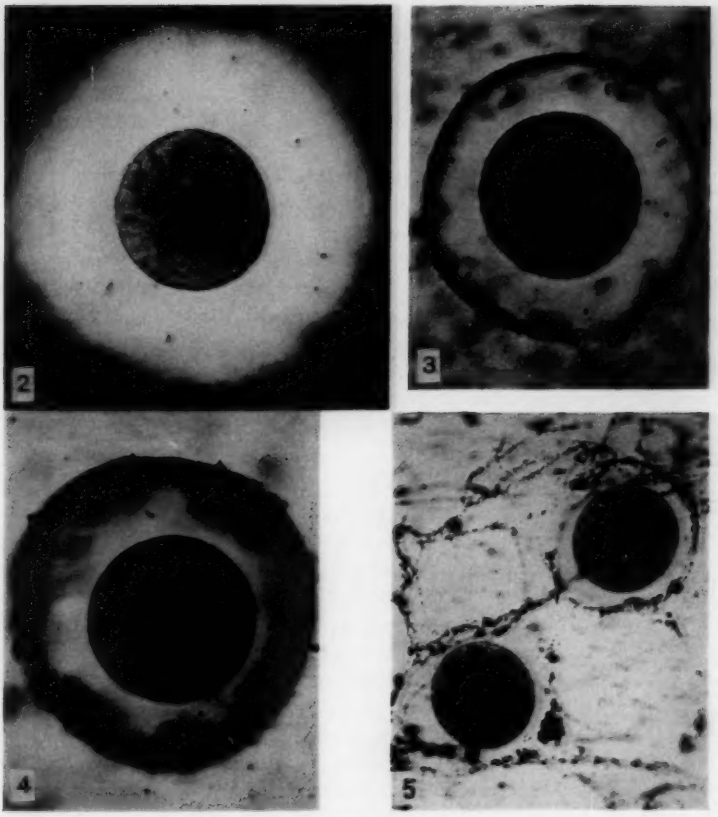

PLATE I

Microscopic appearance of the egg-agglutination reaction. All eggs photographed in Chinese ink.

FIG. 2. Normal *Arbacia* egg in heated sea water surrounded by a wide zone of clear, colorless jelly. × 430.

FIG. 3. A similar egg after five minutes exposure to a 5 per cent sperm extract. Note that a distinct agglutination membrane has appeared at the periphery of the jelly. × 430.

FIG. 4. A similar egg after thirty minutes exposure to a 5 per cent sperm extract. The agglutination membrane has become denser but the jelly immediately in contact with the cortex remains unaffected. × 430.

FIG. 5. Strands of agglutinated jelly connecting eggs in 5 per cent sperm extract. × 215.

a homogeneous suspension which slowly settles (Fig. 1, *C*). Subsequent agitation of each tube causes an increase in the density of the egg-masses in the extract, while the eggs in sea water redistribute themselves through the solution and again settle to the bottom of the tube. We will call this phenomenon the "egg-agglutination reaction," and the inciting substance present in sperm extracts, the egg-agglutinating substance. These terms are used in a purely descriptive sense without implying an analogy to immunological agglutinations.

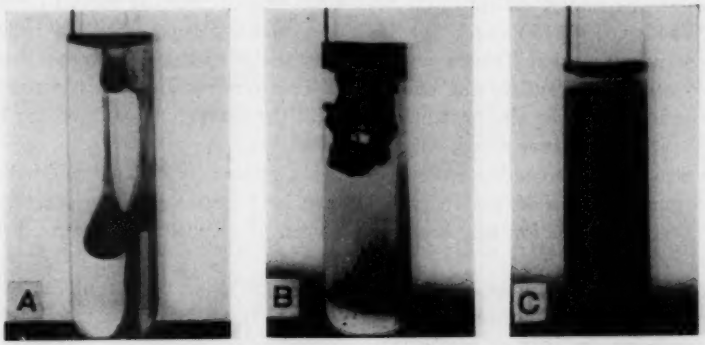

FIG. 1. Gross appearance of the egg-agglutination reaction. All photographs natural size.

(A) Five drops *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. Note the drops of agglutinated eggs suspended from the surface of the solution.

(B) Ten drops of *Arbacia* eggs in 3 cc. of 5 per cent sperm extract. The tube was shaken once. The eggs have formed a large dense mass which floats at the surface.

(C) Ten drops of *Arbacia* eggs in 3 cc. heated sea water. The tube was shaken once after adding the eggs. Observe that the ova are homogeneously suspended and have begun to settle as shown by the clear area immediately below the surface.

The details of the egg agglutination reaction may be observed microscopically by making an egg suspension in Chinese ink which reveals the colorless jelly layer, or chorion, surrounding each ovum. By means of a capillary pipette, sperm extract is introduced into a drop of this mixture mounted on a slide beneath a raised coverslip and observed under high power. As the extract comes in contact with an egg, a faint black line of adhering ink particles immediately appears at the periphery of the jelly. This line rapidly increases in thickness until the chorion is encircled by a dense membranous structure which will be termed the "agglutination membrane."

The microscopic appearance of eggs treated with sperm extract is shown in Plate I, Figs. 2–5. A normal egg is surrounded by a wide zone of clear colorless jelly with poorly-defined margins (Fig. 2). On treating such an egg with sperm extract, a distinct agglutination membrane forms at the periphery of the jelly (Fig. 3), which gradually increases in density (Fig. 4). Particles of jelly freely suspended in the solution also agglutinate to form a dense interlacing network connecting adjacent eggs (Fig. 5).

With these changes the jelly becomes very adhesive, causing the ova to stick firmly together in clusters when the coverslip is moved about. Woodward (1915) states that the jelly of *Arbacia* eggs placed in "boiled sperm suspension" swells and becomes sticky so that the eggs adhere to each other and to the container. This observation was repeatedly confirmed during the present study with the exception that swelling of the jelly was never noted, either in boiled sperm suspensions or in sperm extracts. In some instances the chorion retains its original width but it generally shrinks markedly, as shown by a decrease in the space between agglutination membrane and cortex after prolonged exposure to the solution.

The egg-agglutinating substance affects only that portion of the jelly with which it comes in contact and incomplete agglutination membranes involving one-half or less of the circumference of the chorion appear on those eggs which have been only partially exposed to the extract.

In general, the density and rate of formation of the agglutination membranes are directly related to the concentration of extract. A 4 per cent sperm extract clots the eggs immediately into a solid mass surrounded by a thick unbroken line of agglutinated jelly; as the extract decreases in concentration, fewer eggs are involved, the reaction is slower, and the membranes are thinner until below about 0.5 per cent both gross and microscopic reactions become negative.

The egg-agglutination membranes grow tougher and more resistant to tension with time. After a few minutes exposure to a 2 per cent extract, the coverslip may be gently agitated without altering the circular shape of the membranes and the eggs move about freely in the unaffected central jelly. When greater tension is applied by rapidly agitating the coverslip, the membrane ruptures and the ovum may then be torn away leaving the membrane firmly anchored to the slide with its frayed edges waving about in the solution.

The egg-agglutination reaction is irreversible. Eggs which have once agglutinated remain firmly massed together in spite of transfer to sea water and eventually cytolyse *in situ*. Vigorous shaking increases

the size and density of the egg-clusters and leads to rapid destruction of the ova.

The jelly of *Arbacia* eggs binds the egg-agglutinating substance and removes it from the extract. This is illustrated by a typical experiment: 1 cc. shed eggs was added to 3 cc. of 20 per cent sperm extract and the agglutinated egg-mass was filtered off. The filtrate weakly agglutinated 1 cc. eggs, after which the solution was again filtered but this filtrate failed to agglutinate .5 cc. eggs. Three cc. of the same extract diluted with 2.5 cc. sea water as control strongly agglutinated eggs. The egg-agglutinating substance present in the 20 per cent extract has, therefore, been fixed by the jelly of 2 cc. eggs.

Sperm extracts not only act on the jelly but also on the cortex of *Arbacia* eggs. To study this, jellyless eggs were divided into two lots, one of which was fertilized with fresh sperm. Samples from each lot were fixed in Bouin's solution (without acetic acid) or 2 per cent osmic acid and then washed thoroughly in sea water. Jellyless eggs treated with sperm extract form clusters which are too minute to be reliably detected grossly so a microscopic method was used: a drop of jellyless eggs was mixed with a few drops of extract on a slide and a glass needle was moved through the mixture. The appearance of distinct egg-clusters represented a positive agglutination test.

Numerous experiments demonstrated that the cortex undergoes certain changes in sperm extracts causing the eggs to fuse so firmly together that bits of cytoplasm may be ripped out of the ovum by agitating the egg-clusters. In addition the fertilization membranes of fertilized jellyless eggs adhere to each other and may be pulled completely away from the zygote. These changes take place equally in unfertilized and fertilized jellyless eggs whether living or dead. The structure of the cortex, however, does not appear to be visibly altered by exposure to sperm extracts. Controls of the same eggs in heated sea water remained singly spaced in all cases, except for occasional small clusters caused by the use of acid in removing the jelly (Lillie, 1915*a*).

Identical experiments on normal eggs showed that the jelly of fertilized and unfertilized eggs, both living and dead, agglutinates in sperm extracts. Immature ovocytes, with or without jelly, agglutinate as readily as mature ova.

To sum up: (1) The egg-agglutination reaction is not dependent on living protoplasm, since dead ova agglutinate as readily as living ones. (2) Immature, unfertilized, and fertilized eggs agglutinate equally, indicating that the developmental state of the ovum plays no part in the reaction. (3) Sperm extracts agglutinate jellyless eggs by directly affecting the cortex which becomes markedly adhesive.

One of the first questions to arise was whether the egg-agglutinating substance could be extracted from tissues or cells of *Arbacia* other than sperm. Extracts of *Arbacia* shells, lanterns, peristomes, ovaries, eggs, and coelomic fluid, prepared by heating 4 cc. of each tissue one minute in 10 cc. of sea water, were found to be without effect on ova. Extracts of immature testes or ripe testes washed free of sperm were likewise inert. The egg-agglutinating substance is thus highly tissue-specific and can be extracted solely from sperm. Moreover, sperm extracts act specifically on *Arbacia* eggs and will not agglutinate blood corpuscles, spermatozoa or any other cells of the male or female sea-urchin.

The egg-agglutination reaction is not confined to the gametes of *Arbacia* and a certain degree of cross-agglutination exists between eggs and sperm extracts of related and distant species. Preliminary experiments on the gametes of *Nereis* and *Echinarachnius* gave the following results: (1) *Echinarachnius* sperm extracts agglutinate ova of *Echinarachnius* and *Arbacia*. (2) *Nereis* sperm extracts agglutinate *Arbacia* eggs but do not agglutinate fertilized or unfertilized *Nereis* eggs. In *Nereis*, jelly extrusion occurs after fertilization, hence both fertilized and unfertilized ova were tested for agglutination. Just (1922) found that *Nereis* sperm boiled in sea water causes a small percentage of jelly formation, maturation, and differentiation without cleavage of unfertilized *Nereis* eggs. (3) Sperm extracts of *Arbacia* agglutinate *Echinarachnius* eggs. Such extracts will not agglutinate *Nereis* eggs but cause jelly extrusion and maturation (without cleavage) of unfertilized *Nereis* eggs.

The chemical specificity of the egg-agglutination reaction is less distinct than the biological. *Arbacia* eggs are agglutinated by certain salts, foreign sera (Robertson, 1912), acids and alkalis.

Physical and Chemical Properties of the Egg-Agglutinating Substance

These experiments were devised to study certain general physical and chemical properties of the egg-agglutinating substance and do not represent exact quantitative studies. Each experiment was repeated several times with different materials and the results were consistent in each case. The egg-agglutination tests were standardized by adding three drops of fresh eggs to 2 cc. of sperm extract in a test tube and shaking the tube lightly. In certain experiments the agglutinating strength of the extracts was rated as follows:

0 = No agglutination. All eggs homogeneously suspended.
1 = Few thin shreds of agglutinated eggs with the majority freely suspended.
2 = One or more loose clusters of agglutinated eggs with some ova freely suspended.
3 = Single dense clot of agglutinated eggs with no eggs free in the solution (maximum).

Appearance, Specific Gravity, and pH of Sperm Extracts

Extracts of clean shed sperm are colorless while extracts of testis sperm have a light purple tint ("purple X" of Glaser, 1914). Dilute sperm extracts are clear; those of high concentration (40 per cent) are faintly opalescent. The specific gravity of a 50 per cent extract is 1022, which about equals that of sea water (av. sp. gr. = 1028). The pH of a 60 per cent sperm extract in sea water, measured with the Coleman glass electrode, is 7.74; that of a 1 per cent extract is 8.02. The slight relative acidity of the 60 per cent extract probably results from CO_2 liberated by the thick sperm suspension prior to extraction. These values approximate the pH of sea water at Woods Hole (av. pH = 8.00).

Relation of Temperature to the Extraction and Stability of the Egg-agglutinating Substance

To determine whether the egg-agglutinating substance can be extracted from sperm at low temperatures, 10 cc. sea water was heated in a waterbath to a given temperature, a measured amount of mixed shed sperm was stirred into the sea water and kept for one minute at the desired temperature, after which the solution was filtered. Under these conditions it was found that sea water below about 57° C. will not extract the egg-agglutinating substance. This can be extracted in small amounts from a 20 per cent sperm suspension at 57° and from a 1 per cent suspension at 68° C. Higher temperatures extract greater quantities of the substance from sperm. There appears to be an inverse relation between sperm concentration and the critical temperature of extraction.

The rate of loss of the egg-agglutinating substance on standing is a function of the temperature of the extract: 50 cc. of a 10 per cent sperm extract was divided into two equal parts, one of which was aged at room temperature (26° C.) and the other in an icebox (7° C). The results of tests on each solution at various times are given in Table I.

The agglutinating strength of the extract kept at 7° C. remained constant for twenty-five hours and then gradually diminished and dis-

appeared after forty-five hours. The same extract at 26° C. lost its activity in less than thirteen hours. In a similar experiment, a 1 per cent extract lost its agglutinating power in less than twelve hours at 26° C. and in twenty hours at 7° C. showing that the egg-agglutinating substance disappears more rapidly from dilute extracts. We may conclude that this substance is preserved best at low temperatures.

The egg-agglutinating substance is highly heat-resistant and only slowly destroyed by boiling: 25 cc. of a 20 per cent sperm extract was boiled for several hours in a beaker, the volume being kept constant by addition of distilled water. The agglutinating strength of the extract remained high (3) for four hours, then gradually decreased, and dropped to zero after five and one-half hours of boiling.

TABLE I

Egg-agglutinating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. (3 = maximum)

Age of Extract	Temperature 7° C.	26° C.
hours		
0	3	3
13	3	0
25	3	—
38	2	—
41	1	—
45	0	—

Dialysis and Filtration

Thirty cc. of a 30 per cent sperm extract was divided into three equal parts (*A*, *B*, and *C*), each of which was placed in a Thomas Diffusion Shell (No. 4471). Extract *A* was dialysed against running sea water in a beaker, *B* was suspended in a cylinder containing 10 cc. sea water, and *C* was kept as a control. After twelve hours each extract was tested for its activity. If dialysis had occurred, one would expect a decrease in the concentration of the egg-agglutinating substance in extracts *A* and *B* and the appearance of this substance in the dialysate of extract *B*. By dilution of each solution with sea water, however, it was found that the concentration of the substance in *A* and *B* exactly equalled that of the control, *C*. The dialysate of *B* was totally inert. Numerous similar experiments proved conclusively that the egg-agglutinating substance is non-dialysable. Also it will not pass through a Berkfeld filter regardless of the concentration of sperm extract. These facts suggest that it is a colloidal substance of large molecular size.

Source of the Egg-agglutinating Substance

The sperm heads of *Arbacia* are composed chiefly of nucleic acids and "Arbacin," a substance having properties in common with both histones and protamines (Mathews, 1897). In view of the relatively minute amounts of lipids which are confined largely to the sperm tails, it seemed likely that the egg-agglutinating substance might be a protein or protein derivative. Sperm extracts were found to be negative to the following tests for proteins: Millon's, Adamkiewicz, biuret, xanthoproteic, and ninhydrin. No precipitate forms on adding saturated $(NH_4)_2SO_4$ or 70 per cent alcohol and no reduction occurs with Benedict's solution or bismuth subnitrate. Hence if proteins or carbohydrates are present in sperm extracts, their concentration is too minute to be detected by ordinary chemical tests.

The lipids of sperm may be readily separated from the proteins by alcohol-ether extraction. The procedure used was adapted after Bloor's method for extraction of total lipids from blood serum (Peters and Van Slyke, 1932, p. 495). Ten cc. of mixed shed sperm was shaken in 150 cc. alcohol-ether mixture (three parts 95 per cent alcohol plus one part ethyl ether) and the mixture boiled by immersion in a waterbath. The solution was made up to 200 cc. with alcohol-ether, cooled, and filtered through fat-free filter paper. The rubbery pink protein residue was washed thoroughly in sea water, heated one minute in 10 cc. sea water and filtered. The filtrate strongly agglutinated eggs.

The honey-colored lipid extract was evaporated to dryness on a waterbath, the residue was dissolved in 100 cc. petroleum ether, filtered, and re-evaporated to dryness. This procedure yielded a small quantity of yellow waxy material which was shaken in 10 cc. sea water, heated one minute, and filtered. The filtrate had no effect on eggs.

To determine whether heat was the factor responsible for liberating the egg-agglutinating substance in the above procedure, shed sperm was shaken in cold alcohol-ether solution, after which the protein precipitate was washed in sea water, shaken vigorously in 10 cc. sea water, and filtered. The filtrate markedly agglutinated eggs proving that heat is not essential for extraction.

To sum up, the egg-agglutinating substance is derived from the protein residue (head) of the spermatozoön and is not found in the lipid fraction.

Chemical Composition of the Extraction Medium

Sperm extracts made in tap or distilled water will not agglutinate eggs and if the residue from a sperm suspension, which has been heated

one minute in sea water, is reheated in distilled water, the extract is likewise inert. By substituting sea water for distilled water, in the above procedures, the egg-agglutinating substance is readily obtained. These facts suggested that certain salts or ions, present in sea water, are essential for the extraction of the substance. A thorough study of this problem requires chemical analyses of various extraction media and is beyond the scope of this investigation which is concerned with reactions occurring in sea water. The experiments cited below, therefore, represent preliminary studies:

Ten per cent sperm suspensions were made up in each of the following solutions and extracted by heating one minute and filtering:

1. Van't Hoff artificial sea water solutions each of which lacked a single salt present in normal sea water: $CaCl_2$, $MgSO_4$, $MgCl_2$, KCl, and NaCl.

2. Pure solutions of each of the above salts in distilled water.

3. Pure solutions of Mg, Ca, K, and Na acetate in distilled water.

The reagents were all made up isosmotic with sea water and the pH varied from about 7.2–9.0. (The viscosity, ionic concentration, and molarity could not be controlled.) None of the solutions visibly injured ova or sperm, or agglutinated eggs.

It was found that an active sperm extract agglutinated eggs washed in any of the above media to the same degree as eggs washed in sea water. Shed eggs in sea water were therefore used in the egg-agglutination tests.

From the results, listed in Table II, the following conclusions may be drawn:

(1) Sea water lacking magnesium ($MgCl_2$, $MgSO_4$ or both) will not extract the egg-agglutinating substance from sperm whereas pure solutions of $MgCl_2$ or $MgSO_4$ will. Magnesium is thus essential to the extraction process.

(2) Sea water lacking calcium gave variable results; out of 6 tests, 3 were weakly positive and 3 negative. A pure solution of $CaCl_2$ readily extracts the egg-agglutinating substance. Calcium, therefore, appears to be necessary in sea water for the extraction of this substance.

(3) Sperm extracts made in sea water which lacks either NaCl or KCl agglutinate eggs. Extracts made in pure NaCl or KCl solutions have no effect on eggs. These salts evidently play no part in the extraction of the egg-agglutinating substance in sea water.

(4) If acetate ions are substituted for chloride ions in pure solutions of the salts of sea water, it is found that Mg and Ca acetate will extract the egg-agglutinating substance from sperm but Na and K

acetate will not. The egg-agglutination reaction is therefore not dependent upon the presence of any particular anions.

To sum up, the bivalent cations, magnesium and calcium, are the only ions of *sea water* which are necessary for the extraction of the egg-agglutinating substance from sperm. Both of these ions must be present in sea water but each will act alone when present in sufficient concentration in distilled water.

Extraction of Sperm in Acid and Alkaline Sea Water

In these experiments .10 N HCl and .10 N NaOH were diluted with varying quantities of sea water and the solutions used in preparing 5 per cent sperm extracts.

TABLE II

The agglutination of eggs by 10 per cent sperm extracts prepared in various isosmotic salt solutions. Three drops of eggs added to 2 cc. of each extract. + = positive tests. 0 = negative tests. All solutions prepared by Marine Biological Laboratory Chemical Room.

Extraction Solution	Egg-agglutination
* Artificial Sea Water (Van't Hoff)	+
* Ca-free Sea Water	+ 0
* Mg-free Sea Water	0
* $MgSO_4$-free Sea Water	0
$McCl_2$-free Sea Water	0
KCl-free Sea Water	+
* NaCl-free Sea Water	+
.34 M $CaCl_2$	+
* .37 M $MgCl_2$	+
* $MgSO_4 \cdot 7H_2O$	+
.53 M KCl	0
.52 M NaCl	0
Ca Acetate	+
Mg Acetate	+
K Acetate	0
Na Acetate	0

* Indicates extracts on which fertilizin-inactivation tests were run with results parallel to those for egg-agglutination tests.

Lillie (1915*a*) states that acid sea water heavily agglutinates and later cytolyses *Arbacia* eggs. The present study shows that when sperm is extracted in acid sea water of above .003 N HCl content, the agglutinating effect of the acid hides the action of the egg-agglutinating substance. These substances, however, have different actions since HCl agglutinates and later cytolyses eggs whereas the egg-agglutinating substance agglutinates but does not kill the ova. Sperm extracts in sea water of or below .003 N HCl content agglutinate eggs. Sea water of

this degree of acidity has no effect, which proves that the agglutination is here caused by the egg-agglutinating substance alone.

Haas (1916), Irving (1926), and Kapp (1928) have shown that the addition of .10 N NaOH to sea water precipitates most of the magnesium and some calcium as hydroxides. In the present study it was found that alkaline sea water containing more than .01 N NaOH loosely flocculates *Arbacia* eggs, probably due to precipitation of calcium and magnesium hydroxide in the egg jelly. This precipitate is removed when alkaline sea water is heated and filtered (as in preparing sperm extracts) and eggs added to the filtrate remain singly spaced. It is thus possible to determine the highest concentration of alkali in sea water which will permit the extraction of the egg-agglutinating substance from sperm. This was found to be about .012 N NaOH sea water; above this degree of alkalinity no agglutination takes place; below it the extracts agglutinate eggs. The previous section has shown that sea water lacking Mg or Ca will not extract the egg-agglutinating substance from sperm. According to Irving (1926), .017 molar NaOH precipitates 19 per cent of the total Ca and 16 per cent of the total Mg from sea water. A possible explanation for the action of alkaline sea water in extracting sperm is that sufficient Mg and Ca are removed from sea water by concentrations of alkali above .012 N NaOH to render it inert as an extracting medium. Below this degree of alkalinity, however, enough ionized Mg and Ca are present to allow extraction of the egg-agglutinating substance.

The pH of the solutions varied from 3.2 (.003 N HCl in sea water) to 9.5 (.012 N NaOH in sea water). Since sperm extracts made in either of these solutions agglutinate eggs, the egg-agglutination reaction takes place over a wide range of hydrogen-ion concentrations.

The Inactivation of Fertilizin by Sperm Extracts

As is well known from the classical studies of F. R. Lillie (1913–1919), mature unfertilized eggs of *Arbacia* secrete a substance into sea water which reversibly agglutinates sperm of the same species. Lillie named this substance "fertilizin" and concluded that it functions in fertilization as an essential chemical link in the union of egg and spermatozoön. For further details concerning the fertilizin theory of fertilization see the recent review of Just (1930).

Since fertilizin is present in high concentration in the jelly of *Arbacia* eggs, it seemed probable that sperm extracts might react in some way with this substance, which proved to be correct. When sperm extracts are mixed with egg-water, the latter loses its capacity to agglutinate sperm; some substance in the extract has neutralized the

fertilizin. For description purposes we will call this "fertilizin-inactivation" and the hypothetical substance responsible for it the "fertilizin-inactivator."

To study the relation of fertilizin-inactivation to the concentration of sperm extract, 2 cc. of successive half-dilutions of an 8 per cent extract was placed in a series of test tubes and 2 cc. egg-water of known fertilizin content was added to each tube. This procedure was repeated with egg-waters of descending fertilizin content, the concentration of sperm extracts remaining constant. The highest dilution of egg-water was mixed with an equal quantity of heated sea water as a control. A drop of each mixture was tested for fertilizin by its ability to agglutinate a drop of fresh 1 per cent sperm suspension; absence of sperm agglutination constituted a positive fertilizin inactivation test; agglutination of sperm, indicating the presence of free fertilizin in the mixture, was considered a negative fertilizin-inactivation test.

In Table III, which summarizes the results of three experiments, a change from + to 0 in each horizontal row represents the end-point at which the extract in the corresponding vertical column becomes too dilute to inactivate all of the fertilizin present. For example, 400 fertilizin units are inactivated by a 4 per cent but not by a 2 per cent sperm extract. In each experiment at least three determinations were made at each of these end-points with different sperm suspensions as indicator in order to reduce error caused by variation in materials. Note (Table III) that an 8 per cent extract inactivates 800 fertilizin units whereas a .06 per cent extract only inactivates 10; a .1 per cent extract inactivates 20 but not 40 fertilizin units. The controls agglutinated sperm in all instances. Sperm extracts thus inactivate fertilizin in roughly quantitative proportions, the capacity for inactivation varying directly with the concentration of extract.

Certain incidental phenomena were noted during the course of this experiment. When a drop of concentrated sperm extract plus egg-water is added to a drop of spermatozoa, the latter form a dense, highly active ring around the introduced drop but sperm agglutination does not occur. Sperm extracts therefore react only with fertilizin without altering the "sperm-activating and aggregating" substances of egg-water, which confirms Lillie's statement (1914) that these substances are distinct from fertilizin. Furthermore, it was noted that on shaking concentrated sperm extract plus egg-water mixtures, a light purple, flocculent precipitate appears and the egg-water, which previously was purple, immediately becomes colorless. The precipitate floats at the surface of the solution and is probably formed by agglutination of sub-microscopic jelly particles present in the egg-water.

It may be pointed out here that fertilizin does not appear to be essential to the egg-agglutination reaction, since fertilized jellyless eggs washed free of fertilizin agglutinate as readily as unfertilized jellyless eggs which secrete large quantities of this substance.

TABLE III

Combined results of three experiments on the inactivation of fertilizin by sperm extracts. Upper horizontal column gives concentration of sperm extracts; vertical column gives concentration of egg-water in units of fertilizin. For each test, 2 cc. egg-water was added to 2 cc. extract and the mixture tested for its ability to agglutinate a 1 per cent sperm suspension. + = positive tests (no sperm agglutination); 0 = negative test (sperm agglutination).

Concentration of Sperm Extract:	8%	4%	2%	1%	0.5%	0.2%	0.1%	0.06%	0.03%
Fertilizin Units	Fertilizin-Inactivation Tests								
800	+	+	0	0	0				
	+	+	0	0	0				
	+	+	0	0	0				
400	+	+	0	0	0	0			
		+	0	0	0				
200	+	+	+	0	0	0			
		+	+	+	0	0			
			+	+	0	0			
100		+	+	+	0	0			
		+	+	+	0	0			
80			+	+	+	0	0		
			+	+	+	0	0		
			+	+	0	0	0		
40			+	+	+	0	0		
			+	+	+	+	0		
				+	+	0	0		
20				+	+	+	+	0	
				+	+	+	+	0	
				+	+	0	0	0	
10					+	+	+	+	+
					+	+	+	+	0

PROPERTIES OF THE FERTILIZIN INACTIVATOR

We have described two effects of sperm extracts, one on eggs and the other on fertilizin. To determine whether these effects are related

to each other, simultaneous tests for the egg-agglutinating substance and fertilizin-inactivator were run on sperm extracts. The fertilizin-inactivation tests represent a part of the previously described experiments dealing with the properties of the egg-agglutinating substance and the results of the former will, therefore, be briefly summarized without repeating experimental details. The fertilizin-inactivation tests were standardized by adding .3 cc. sperm extract to .3 cc. egg-water of known fertilizin content and testing the mixture for agglutination on a fresh 1 per cent sperm suspension. The inactivating strength of a sperm extract was considered equal to the maximum number of fertilizin units which it neutralized.

The fertilizin-inactivator is markedly tissue-specific and cannot be extracted from any tissue or cells of *Arbacia* except sperm. It has long been known that blood and tissue extracts of *Arbacia* will not combine with fertilizin but that extracts of washed unfertilized eggs contain a substance, "anti-fertilizin," which neutralizes minute amounts of fertilizin (Lillie, 1914). Because anti-fertilizin combines only with fertilizin without affecting the sperm-aggregating and activating factors of egg-water, it acts like the fertilizin-inactivator, but the latter is much more potent in neutralizing fertilizin.

TABLE IV

Fertilizin-inactivating strength of a 10 per cent sperm extract aged at 7° C. and 26° C. Inactivating strength given as maximum number of fertilizin units inactivated by extract.

Age of Extract	Temperature 7° C.	26° C.
hours		
0	400	400
13	400	50
25	200	—
38	100	—
41	50	—
45	25	—

Low temperatures preserve the fertilizin-inactivator as shown in Table IV, which gives the data from a single experiment. After thirteen hours at 26° C. the inactivating strength of a 10 per cent extract dropped from 400 to 50; after twenty-five hours at 7° C. the inactivating strength fell from 400 to 200 and then gradually decreased to 25 in forty-five hours.

The fertilizin-inactivator is quite thermostable and is gradually destroyed by boiling. The inactivating strength of a 20 per cent sperm

extract fell from 400 to 50 after two and one-half hours at 100° C. and remained at 50 after five and one-half hours of boiling.

It can be obtained from the protein residue but not from the lipid fraction when sperm is extracted with hot alcohol-ether and it is extractable from sperm shaken in cold alcohol-ether. It will not dialyse or pass through a Berkfeld filter and is not present in sperm extracts made in tap or distilled water. In the previously described experiments with artificial sea water solutions, fertilizin-inactivation tests were run on a few of the extracts (marked with a (*) in Table II) and the results paralleled the egg-agglutination tests in each case.

Because the egg-aggglutinating substance and fertilizin-inactivator in general showed parallel behavior in all experiments they will be referred to from now on collectively as the "active principles" of sperm extracts.

Secretion of Active Principles and their Relation to the Fertilizing Power of Sperm

Filtrates of fresh or old sperm suspensions which have been passed through Whatman filter paper and the supernatant fluid of centrifuged sperm suspensions will not react with fertilizin or eggs. The active principles are found only in sperm *extracts* and are not secreted by sperm into water.

It was found that extracts of sperm which have been aged until dead are inert. The fact that the active principles can only be extracted from fresh sperm suggested that they might be related to the fertilizing power of sperm. Experiments were therefore undertaken to determine whether the loss of fertilizing power of aging sperm suspensions bears any relation to the loss of active principles from extracts of these suspensions. Sperm suspensions of various concentrations (8 per cent, 4 per cent, 2 per cent, and 1 per cent) were prepared by adding measured amounts of mixed shed sperm to 80 cc. sea water in a series of Erlenmeyer flasks. The sea water was sterilized in an autoclave to lessen bacterial growth which shortens the life of sperm (Cohn, 1918). The flasks were closed with sterile corks and aged at room temperature (26°–28° C.) for three days. At intervals 4 cc. of each suspension was heated thirty seconds, filtered, and the extract divided into two equal parts which were tested for the active principles.

In the fertilizin-inactivation tests, in order to insure complete inactivation of fertilizin by each extract at the start of the experiment, descending concentrations of fertilizin were added; 200 fertilizin units were added to the 8 per cent, 100 to the 4 per cent, 50 to the 2 per cent and 25 to the 1 per cent sperm extract.

Simultaneously with these tests, the fertilizing power of the sperm was measured by adding one drop from each sperm suspension to three drops of fresh eggs in 10 cc. sea water and counting the percentage of fertilized eggs in each dish two hours later.

TABLE V

The relation between loss of fertilizing power of aging sperm suspensions and loss of active principles from extracts of these suspensions. Horizontal rows give simultaneous tests made at intervals recorded at left. *A* = egg-agglutination tests. *B* = fertilizin inactivation tests; the figures in brackets are the number of fertilizin units added to each extract. *C* = fertilizing power of sperm suspensions expressed as the percentage of eggs fertilized by each suspension. + = positive tests; 0 = negative tests.

Age of Sperm Suspensions	*A* Sperm Extracts: Egg-agglutination Tests				*B* Sperm Extracts: Fertilizin-inactivation Tests				*C* Sperm Suspensions: Percentage of Eggs Fertilized			
					(200)	(100)	(50)	(25)				
	8%	4%	2%	1%	8%	4%	2%	1%	8%	4%	2%	1%
hours												
24	+	+	+	0	+	+	+	+	100	100	100	100
30	+	+	+	+	+	+	+	+	100	99	100	89
36	+	+	+	0	0	+	+	+	100	100	100	94
49	+	+	0	0	+	+	0	0	100	93	12	11
56	+	+	0	0	+	+	0	0	100	13	0	3
72	0	0	0	0	0	0	0	0	26	0	0	0

The data are recorded in Table V. Column *A* lists the egg-agglutination tests, column *B* the fertilizin-inactivation tests, and column *C* the fertilizing power of each suspension. Each horizontal row thus records comparable tests at various times. For the first thirty-six hours the active principles are present, in general, in all extracts except for the 1 per cent which has lost its capacity to agglutinate eggs. Throughout this period the sperm suspensions fertilize 89–100 per cent of the eggs. After forty-nine hours, both egg-agglutination and fertilizin-inactivation tests are negative in the 2 per cent and 1 per cent extracts and at this time the fertilizing power of the 2 per cent and 1 per cent sperm suspensions has dropped to 12 per cent and 11 per cent respectively. The active principles have disappeared in all extracts after seventy-two hours and the fertilizing power of each suspension has dropped to zero except for the 8 per cent suspension which fertilizes

26 per cent of the eggs. At the close of the experiment living sperm were present in each suspension (except the 1 per cent) as shown by clear cytoplasm of the sperm heads and slight motility.

Many sources of error are present in an experiment of this sort which cannot be adequately controlled. The necessity for testing the same solutions for three days introduces unavoidable contamination and the condition of the eggs and sperm used for testing varies widely in different urchins from day to day (Goldforb, 1929). Nevertheless, it is clear that the loss of fertilizing power of aging sperm suspensions parallels the loss of active principles from extracts of these suspensions.

The Effect of Sperm Extracts on Fertilization and Development

Effect of Sperm Extracts on Both Gametes

In an extensive series of experiments, normal and jellyless eggs were fertilized in sperm extracts (.1 per cent to 8 per cent), transferred to sea water after exposure to the extracts for from thirty seconds to three hours, and at various times counts were made of the percentage of eggs developing. Controls consisted of the same eggs fertilized in heated sea water. Fertilization and development in extracts below about 2 per cent concentration equals that of the controls but above this concentration marked inhibition occurs. The majority of ova remain unfertilized and no fertilization membranes appear despite the presence of numerous active sperm at the cortex. Those eggs which are activated usually cleave normally but cease developing at or before the non-motile blastula stage if allowed to remain in the extracts. When the embryos are transferred to sea water they develop into normal plutei. These effects occur in both jellyless and normal eggs but the inhibition is greater in the latter, probably due to agglutinated jelly which physically prevents escape of the blastulae from the egg-masses.

In these experiments both germ-cells were simultaneously exposed to sperm extracts and the block to fertilization could be due to the action of extracts on the ovum alone, spermatozoön alone or on both gametes. We will now consider the action of extracts on each gamete.

Effect of Sperm Extracts on the Fertilizing Power of Sperm

Sperm suspensions were made in sperm extracts and fresh eggs fertilized with these mixtures: 1 per cent sperm suspensions were prepared in .5 per cent to 8 per cent extracts and after exposure of sperm to the extracts for various times, one drop from each suspension was used to fertilize three drops of fresh eggs in 20 cc. sea water. The per-

centage of fertilized eggs was counted two hours after insemination. A 1 per cent sperm suspension in boiled sea water served as control.

TABLE VI

The inhibiting effect of sperm extracts on the fertilizing power of sperm. One per cent sperm suspensions prepared in sperm extracts of various concentrations. Figures give percentage of fresh eggs fertilized by one drop from each suspension after exposure of sperm to the sperm extracts for times given at left. Control = 1 per cent sperm suspension in boiled sea water.

Concentration of Sperm Extracts	8%	4%	1%	0.5%	Control
Time Sperm Exposed to Sperm Extracts	Percentage Eggs Fertilized				
5 seconds.....	18	1	68	—	
15 seconds.....	3	2	20	92	
45 seconds.....	8	3	17	100	
60 seconds.....	7	5	18	92	
15 minutes.....	3	3	32	12	
30 minutes.....	3	1	5	4	99
60 minutes.....	1	1	1	1	99

From Table VI, which gives the result of one experiment, it is seen that the percentage of eggs fertilized by sperm suspensions in extracts of above 1 per cent drops greatly after fifteen seconds at which time the 4 per cent suspension fertilizes only 2 per cent of the ova. After fifteen minutes exposure, the fertilizing power of the .5 per cent suspension has dropped from 92 per cent to 12 per cent; after thirty minutes the percentage of eggs fertilized by each suspension has fallen almost to zero. The controls fertilized 99 per cent of the ova after one hour. Numerous motile sperm were seen at the surface of the ova but fertilization membranes did not appear. Sperm extracts, then, block fertilization by a rapid inhibiting effect on sperm which lose their fertilizing power but retain motility. The loss of fertilizing power is directly proportional to both the concentration of extract and the time of exposure.

Effect of Sperm Extracts on the Fertilizability of Eggs

These experiments were run on jellyless eggs in order to permit direct contact between cortex and sperm extract and to eliminate any mechanical interference with fertilization due to agglutination of the egg jelly. Twenty drops of jellyless eggs from a single female were placed in 10 cc. sperm extracts (.1 per cent to 8 per cent) and at intervals ten

drops of eggs were transferred from each extract to 10 cc. sea water. The ova were fertilized immediately after transfer by two drops of a 1 per cent sperm suspension (made up fresh at the start of the experiment and used throughout) and the percentage of eggs developing was counted two hours later. Twenty drops of the same eggs in boiled sea water were treated in the same manner and served as control.

TABLE VII

The inhibiting effect of sperm extracts on the fertilizability of eggs. Jellyless eggs added to sperm extracts of various concentrations, transferred to fresh sea water, and immediately inseminated with fresh sperm. Figures give percentage eggs fertilized after exposure to the extracts for the times given at left. Control = same eggs in boiled sea water.

Concentration of Sperm Extracts	8%	2%	0.5%	0.1%	Control
Time Jellyless Eggs Exposed to Sperm Extracts	Percentage Eggs Fertilized				
minutes					
15	20	57	91	91	
30	24	45	90	98	
60	15	17	89	96	100
90	21	21	87	97	
120	2	8	66	93	92

The data of a typical experiment are given in Table VII. After two hours exposure to extracts of above 2 per cent, less than 10 per cent of the eggs are fertilized. At this time less inhibition occurs in the eggs exposed to the .5 per cent extract since 66 per cent are fertilized. The .1 per cent extract equals the controls (91–98 per cent fertilized). We may conclude that the inhibiting effect of sperm extracts on the fertilizability of eggs is due to an effect on the cortex and is directly related to the concentration of extract and length of exposure.[2]

Effect of Sperm Extracts on Fertilized Eggs and Embryos

We have seen that fertilization and development of eggs fertilized in sperm extracts are inhibited when the gametes are exposed to extracts

[2] Glaser (1914) stated that eggs which have been treated for two hours with "boiled sperm infusion" remain unfertilized when heavily inseminated. He believed this inhibition to be due to "purple X" since boiled sperm infusions from which the color had been removed did not block fertilization. Woodward (1915) recorded similar results. In the present study it was found that the presence of coloring matter did not appear to influence in any way the inhibiting action of sperm extracts on the fertilizability of eggs.

during their union in fertilization. The inhibiting effect of sperm extracts also occurs *before* the union of the germ-cells, involving egg and sperm individually. We will now consider the action of sperm extracts on eggs *after* normal fertilization.

Jellyless and normal eggs were inseminated in sea water, transferred to sperm extracts (8 per cent to .1 per cent) ten minutes after fertilization, and some of the eggs were transferred back to sea water after exposure to the extracts for from thirty seconds to one hour. Controls were the same eggs in sea water. The following observations were made:

Three hours after fertilization—Normal cleavage in all extracts equal to that of controls.

Seven hours after fertilization—Development of eggs in extracts of above 2 per cent concentration stopped at non-motile blastula stage. Healthy motile blastulae found in remaining extracts and controls.

Twenty-three hours after fertilization—Embryos in 8 per cent extract have cytolysed at the blastula stage. Rare plutei in the 2 per cent extract with majority cytolyzed at blastula stage. Healthy plutei found in remaining extracts and controls.

Throughout the series the ova transferred to sea water developed into normal plutei, indicating that the inhibitory effect of sperm extracts is reversible. In the concentrated extracts normal eggs were inhibited to a slightly greater degree than jellyless eggs due to agglutinated egg-masses preventing escape of the blastulae.

When a drop of sperm extract is added to a drop of free-swimming plutei in Chinese ink, the embryos are paralysed at once on entering the extract and fall to the bottom. Peristaltic movements of the gut and feeble ciliary motion are the only indications that the embryos are alive. Under high power the cilia are stuck together by their tips and the plutei are surrounded by clumps of ink particles enmeshed in the agglutinated cilia. Due to the increased stickiness of the cilia, the plutei adhere together in large clusters when a glass needle is moved through the solution. This phenomenon occurs in plutei which have developed from completely jellyless eggs and so is not the result of agglutination of particles of egg jelly by the sperm extract.

In attempting to explain these findings it must be realized that extracts of whole sperm undoubtedly contain numerous unknown substances but the action of the egg-agglutinating substance accounts for many of the above facts. We have seen that eggs fertilized in sperm extracts or placed in extracts after fertilization in sea water proceed

normally to the non-motile blastula stage and then abruptly stop developing. It is likely that the cilia, which are pushed forth from the surface of the blastula at this time, are immediately agglutinated by the extract with the result that the blastulae are unable to break out of the fertilization membranes and die *in situ*. When embryos are transferred to sea water from the extract, the cilia are able to function, motility follows, and development continues normally. Paralysis of motile embryos by sperm extracts is probably also caused by agglutination of the cilia.

Parthenogenesis with Sperm Extracts

Repeated attempts were made to initiate development in unfertilized *Arbacia* eggs by exposing them to sperm extracts. Both normal and jellyless eggs were immersed in extracts of various concentrations (.5 per cent to 100 per cent) for from five seconds to twenty-four hours and then transferred to sea water. In other experiments eggs were treated with mixtures of egg-water and sperm extracts, extracts from which the active principles had been removed by boiling or aging, dialysed extracts, etc. The results were entirely negative in all instances. It is clear that immersion of eggs in sperm extracts is not sufficient stimulus for development. Perhaps local application of sperm extracts may be effective.

Discussion

We have seen that there are two major effects of sperm extracts, one upon the surface of the egg and the other a reaction with fertilizin, which paralleled each other under various experimental conditions. It is not possible to determine whether these effects represent the activity of two distinct substances or two properties of a single substance since the indicators for each are different; eggs for the egg-agglutinating substance and fertilizin for the fertilizin-inactivator. It will be assumed that a single "active substance" is present in sperm extracts which reacts with eggs and fertilizin.

The question of chief importance relating to this study is whether the active substance functions in fertilization. This substance is extractable only from sperm, agglutinates *Arbacia* eggs and embryos but has no effect on *Arbacia* sperm or blood corpuscles, and combines specifically with fertilizin which is believed to play an important part in fertilization. This marked degree of tissue specificity between eggs, fertilizin, and the active substance suggests that the latter is related to fertilization.

Because sperm loses its fertilizing power before loss of motility, Lillie (1915*b*) postulated that sperm contains a "fertilizing substance"

which combines with fertilizin and thereby activates the ovum. Such a substance, if present in sperm extracts, must theoretically (1) combine with fertilizin and neutralize its capacity to agglutinate sperm, (2) be lost with the fertilizing power of sperm, and (3) activate unfertilized eggs. Since sperm extracts inactivate fertilizin in quantitative proportions and loss of fertilizing power of sperm suspensions parallels loss of the active substance from extracts of these suspensions, two of the above criteria are fulfilled. On the other hand, sperm extracts will not activate eggs, which would tend to disprove the similarity of the fertilizing and active substances. It is possible that the active substance is non-diffusible and hence cannot permeate the cortical membrane, local application is necessary to cause activation, or a penetrating force (sperm) is essential to carry the fertilizing substance into the cytoplasm. Fertilization might then result from injecting sperm extracts into the cortex or increasing the permeability of the vitelline membrane. The block to fertilization caused by sperm extracts is additional evidence against the identity of the active and fertilizing substances. Assuming that fertilizin is necessary for activation, the inhibiting effect of sperm extracts on the fertilizability of eggs may be due to the neutralization of fertilizin at the egg surface by the active substance of sperm extracts. Possibly agglutination of the peripheral layer of the cortex physically prevents sperm penetration.

Lillie (1919, p. 219) suggests that there is a high degree of chemical specificity in the union of egg and sperm and Just (1930, p. 331), defending Lillie's use of immunological terms in his fertilizin theory, states, ". . . The biology of fertilization has more points in common with immune reactions than with any other biological phenomenon." The active substance of sperm extracts resembles bacterial agglutinins in certain respects. The following properties of bacterial agglutinins are taken from Wells (1929) and Kolle and Hetsch (1935): (1) Agglutinins disintegrate in sera when preserved, are non-dialysable, pass only incompletely through Chamberland filters, and are not destroyed by extracting serum with lipoid solvents. (2) Electrolytes are necessary for agglutination reactions, which will not occur in distilled water. The cation and not the anion of the added salt is of importance. The reactions take place over a wide range of hydrogen-ion concentration (pH 3.7–9.0). (3) The specificity of agglutination reactions is less marked than that of other immune reactions due to the complex structure of cells which permits similar proteins, carbohydrates and lipids to occur in cells of different species; different species of bacteria will thus react with a single agglutinin. (4) Dead bacteria agglutinate as readily as living ones and the agglutination of living bacteria does not kill them. In each

of the above properties there is a remarkable similarity between the agglutination of bacteria by sera and the agglutination of eggs by sperm extracts. Certain differences are also found; thus all known agglutinins are destroyed at or below 70° C. whereas the egg-agglutinating substance resists boiling for hours.

One of the theories concerning the mechanism of agglutination is that the agglutinin reduces the electronegative charge which in part keeps bacteria (or other cells) in suspension, thereby rendering them more susceptible to the precipitating action of salts. We have pointed out that egg-agglutination occurs only in the presence of certain cations (Mg or Ca) and according to Heilburnn (1923), the surface of *Arbacia* eggs is negatively charged. Possibly the active substance of sperm extracts reduces this negative charge at the surface of the ovum and permits the cations of the electrolyte to precipitate the eggs. The agglutination membrane would then form by precipitation of dissolved colloids at the periphery of the jelly, which has been "sensitized" to the precipitating action of salts by the active substance.

The active substance appears to be related to fertilizin in that both substances are non-dialyzable, thermostable, colloidal, and do not give the usual protein tests. It is likely that the active substance and fertilizin belong to a class of chemical compounds related to, if not identical with, bacterial agglutinins.

In conclusion, the remarkable tissue specificity of the active substance, its ability to combine selectively with fertilizin, its agglutinating action on eggs and embryos, its relation to the fertilizing power of sperm, and finally, the analogies between immune reactions, fertilization, and the properties of the active substance, all suggest that this substance plays a definite rôle in the fertilization reaction.

Summary

1. Sperm extracts of *Arbacia*, prepared by heating and filtering sperm suspensions in sea water, irreversibly agglutinate eggs of the same species. The reaction is characterized grossly by a dense coagulation of the ova and microscopically by the appearance of an "agglutination membrane" at the periphery of the egg jelly. Sperm extracts agglutinate both jelly and cortex of unfertilized, fertilized, living or dead ova.

2. The "egg-agglutinating substance" is fixed by the jelly and removed from solution. It is found only in extracts of sperm and cannot be extracted from any other cells or tissues of the sea-urchin, is not secreted by sperm into sea water, and is not present in extracts of old sperm. The egg-agglutinating substance is colorless, non-dialysable,

colloidal, highly thermostable, disintegrates on standing, is preserved best at low temperatures, and does not give the usual protein tests. On extracting sperm with hot or cold alcohol-ether, the egg-agglutinating substance is found in the protein residue but not in the lipid extract. Mg and Ca ions are the only ions of sea water which are essential to the extraction of the egg-agglutinating substance, no specific anions taking part in the process. Sperm extracts made in distilled or tap water are inert.

3. Sperm extracts inactivate the sperm-agglutinating power of *Arbacia* fertilizin, the capacity for inactivation varying directly with the concentration of extract. The " fertilizin inactivator " has many properties in common with the egg-agglutinating substance. Loss of fertilizing power from aging sperm suspensions parallels the loss of the egg-agglutinating substance and fertilizin inactivator from extracts of these suspensions.

4. Sperm extracts block fertilization in *Arbacia* by a direct effect on each gamete:

(*a*) Sperm suspensions in sperm extracts rapidly lose their fertilizing power as measured by their ability to fertilize fresh eggs in sea water. The extracts thus block fertilization by a direct action on the spermatozoön.

(*b*) The fertilizability of jellyless eggs is greatly decreased after exposure to sperm extracts, which exert an inhibitory effect on the cortex.

(5) *Arbacia* embryos are instantly paralysed and agglutinated by sperm extracts. Eggs which are fertilized in extracts cease developing before the motile blastula stage but this effect is partially removed by transferring the eggs to sea water.

6. All attempts to activate *Arbacia* eggs by immersion in sperm extracts have been unsuccessful.

7. It is suggested that the agglutination of eggs and the inactivation of fertilizin by sperm extracts represent two effects of a single active substance extracted from sperm. Theoretical and experimental evidence is presented that this substance probably plays a definite rôle in the fertilization reaction.

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EFFECT OF CERTAIN BACTERIA ON THE OCCURRENCE OF ENDOMIXIS IN PARAMECIUM AURELIA[1]

ARLENE JOHNSON DE LAMATER

(From the Zoölogical Laboratory, Johns Hopkins University)

It was at first believed that endomixis occurs in *Paramecium aurelia* at rather regular intervals, but detailed observations have shown that there is much variation in the length of the interendomictic period. Different stocks show differences in the average interval between endomixes: thus Sonneborn (1937) showed that, under identical conditions, in the stock *R* the mean interval between endomixes was 18.3 days (66.3 fissions), while in Woodruff's long-lived stock (*W*) the mean interval was 46.3 days (110.4 fissions). Environmental conditions have been shown to alter the interendomictic period. Woodruff (1917) showed that sudden alterations in the cultural conditions bring on endomixis earlier than it would otherwise occur. Jollos (1916), by various changes in temperature and bacterial content of the culture medium, caused great changes in the length of the interendomictic period, from a minimum of 3 days to a period sufficiently great for 168 fissions to occur. Young (1917) increased the frequency of endomixis in *Paramecium aurelia* by alterations in the age of the culture medium and by changes in temperature. In *Paramecium caudatum,* Chejfec (1930) showed that the percentage of individuals undergoing endomixis in mass cultures was greater in a medium containing the bacillus coli than in hay infusion.

In the following study of *Paramecium aurelia* the frequency of endomixis and length of the interendomictic period were compared in culture media containing diverse bacteria, in different concentrations. Special attention was directed to the question whether it is primarily the unfavorable nature of conditions that brings on endomixis or increases its frequency.

A branch of Woodruff's long-lived stock of *Paramecium aurelia* was employed in the experiments. All lines of descent were derived from a single individual which underwent endomixis at room temperature during the first five-day period of the experiment (see Fig. 1). Thereafter all cultures were kept in a constant temperature box at about 25° to 26° C., except during the daily transfers.

[1] I wish to thank Dr. H. S. Jennings and Dr. T. M. Sonneborn for their help and criticism.

Five types of bacteria were employed in the different culture media, namely *Flavobacterium brunneum, Bacillus niger, B. cereus, B. prodigiosus,* and *B. coli.*

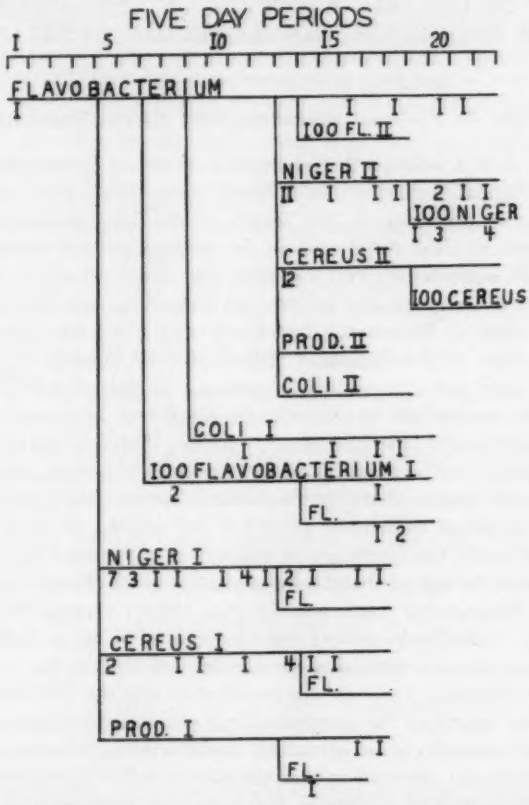

FIG. 1. Diagram of the course of the experiments. Each interval from left to right represents a five-day period. The horizontal lines show the number of five-day periods through which each culture (of 12 lines each) continued. The small numbers below the horizontal lines in certain periods give the number of lines (out of the 12) that underwent endomixis in that period. The heavy vertical lines show the source of the cultures; thus the culture "Niger I" was derived from the *Flavobacterium* culture at the end of its fourth five-day period: it continued through the seventeenth five-day period.

Flavobacterium, Flavobacterium brunneum; Niger, Bacillus niger; Cereus, B. cereus; Prod., B. prodigiosus; Coli, B. coli.

Designations preceded by 100 indicate cultures in which the concentration of bacteria was 100 times as great as normal; designations not preceded by 100 indicate the normal concentration. I and II indicate Groups I and II, mentioned in the text.

The standard culture medium was that commonly employed in this laboratory. It consists of infusion of desiccated lettuce leaves, into which is introduced the green alga *Stichococcus bacillaris,* in the proportion of three 2 mm. loops of the alga (from 20 to 22-day-old slants) to 20 cc. of the filtered fluid. To this is added a 1 mm. loop of *Flavobacterium brunneum* from a one-day-old agar slant. (For details see Sonneborn, 1936.) Since *Paramecium aurelia* flourishes well in this medium, it was employed as a norm or standard for comparing the results with the other bacteria.

For the other bacteria the medium was prepared in the same manner, with the substitution of equal amounts of the other bacteria for *Flavobacterium brunneum.* The medium is buffered to prevent the production of different pH by the diverse bacteria. In some of the experiments a nutritive medium was employed in which the quantity of bacteria was 100 times as great per unit volume as in the normal or standard medium. The purpose was to determine the results of preponderance of bacteria of a certain type, so that it was not considered essential to carry on the experiments under purely aseptic conditions; this would, for experiments of this nature, be practically impossible. Tests were made at intervals to determine whether there was marked contamination. No cross-contamination of the different bacterial types employed was observed in any case. There were three types of common laboratory contaminants; when these became at all abundant in the cultures (up to 30 per cent) such cultures were rejected.

For determining how far the effects of certain bacteria in certain concentrations are harmful or unfavorable, the following criteria were used. An unfavorable effect may be shown by (1) a slowing of the fission rate, (2) an increase in the proportion of deaths occurring, (3) incomplete division, resulting in the production of partially united chains of individuals. Accordingly, observations on these points were recorded for each different type of culture (see Table I). The occurrence of endomixis in the different media was detected by the daily staining of samples from each line.

The general plan of the experiments is shown in Fig. 1, on which is likewise indicated the number of lines in which endomixis occurred in particular five-day periods. In Table I are summarized the general results of culture in the different media. The following account of results is to be read in connection with the figure and table.

From a single individual that had just undergone endomixis, twelve lines were developed as quickly as possible, by cultivation in the normal *Flavobacterium* medium described above. This culture of 12 lines in *Flavobacterium* was maintained throughout the 22 five-day periods of

TABLE I

A summary of the effects of different bacterial media upon endomixis, the number of deaths and of chain-formation in Paramecium aurelia

Group	Total number of line-days	Mean fissions per line per five-day period	Total number of deaths	Number of deaths per 100 line-days	Total number of chains formed	Number of chains per 100 line-days	Total number of lines in endomixis	Number of lines in endomixis per 100 line-days
Flavobacterium brunneum								
Normal Concentration	1232	10.38	10	.81	2	.16	5	.41
100 Times Normal (Group I)	748	5.27	31	4.14	26	3.48	3	.40
Group I Returned to Normal	360	9.13	3	.83	3	.83	3	.83
100 Times Normal (Group II)	236	6.09	9	3.81	9	3.81	0	0
Bacillus niger								
Normal Concentration (Group I)	746	8.23	6	.80	0	0	23	3.08
Group I Returned to *Flavobacterium*	291	11.92	0	0	0	0	0	0
Normal Concentration (Group II)	590	8.91	9	1.53	0	0	18	3.05
100 Times Normal	240	9.82	3	1.25	0	0	8	3.33
Bacillus cereus								
Normal Concentration (Group I)	750	8.69	4	.53	0	0	11	1.47
Group I Returned to *Flavobacterium*	220	8.88	0	0	0	0	0	0
Normal Concentration (Group II)	297	8.93	3	1.01	0	0	12	4.04
100 Times Normal	225	4.61	7	3.11	7	3.11	0	0
Bacillus prodigiosus								
Normal Concentration (Group I)	772	8.88	7	.91	7	.91	2	.26
Group I Returned to *Flavobacterium*	207	8.48	0	0	2	.97	0	0
Normal Concentration (Group II)	197	9.42	4	2.03	4	2.03	1	.51
Bacillus coli								
Normal Concentration (Group I)	658	9.43	15	2.28	15	2.28	4	.61
Normal Concentration (Group II)	359	8.92	8	2.23	10	2.79	0	0

the experiments; it is represented by the upper horizontal line in Fig. 1. Cultures employed in the experiments with other media were branched off from this normal culture in particular five-day periods, as shown in Fig. 1. In each of the different media a group of 12 lines was kept in progress during a number of five-day periods, as indicated in the figure. The number of "line-days" given in column 2 of Table I is the number of lines of descent (usually 12, but with occasional irregularities), multiplied by the number of days during which the lines were in progress. In some cases two different groups of 12 were tested at different times in a particular medium; these are designated as I and II in the figure and table.

The main features of the diverse experimental cultures are as follows.

FLAVOBACTERIUM BRUNNEUM: NORMAL OR STANDARD CONCENTRATION

The 12 lines in this medium were kept in progress for 22 five-day periods, which covered the entire time of the experimental cultures. Total number of line-days, 1232. Mean fission rate, 10.38 per line per five-day period. Endomixis had occurred at the beginning of the culture; it occurred again in but four of the lines, in periods 16, 18, 20 and 21 (Fig. 1)—after intervals respectively of 75, 87, 97 and 103 days (168, 180, 212 and 224 generations). The remaining 8 lines did not undergo endomixis during the rest of the 110 days of the culture. The interendomictic periods are thus very long (as was before known to be the case in this stock). Thus, as the table shows, the total number of lines found to be in endomixis during this part of the experiment was but 5 (including that in the first generation), so that the number of endomictic lines per 100 line-days was but 0.41 (Table I).

In this medium the total number of deaths was but 10, or in the proportion of 0.81 per 100 line-days. Only 2 cases of united chains (incomplete division) occurred, thus but 0.16 per 100 line-days.

High concentrations of *Flavobacterium*.—At the beginning of the seventh 5-day period a group of 12 lines (Group I) was branched off and cultivated in the same medium, but with the concentration of bacteria 100 times as great. They were continued here till the end of the 19th period. As Table I shows, this decreased the fission rate to about one-half its former rate (5.27 in place of 10.38 per five-day period): multiplied the death rate by five and the proportion of incomplete fissions (chains) by about 22. Furthermore, the size of the individuals was notably decreased, and the contained crystals became larger and more numerous. Thus this high concentration must be considered distinctly unfavorable as compared with the normal. Seven days after the trans-

fer (in period 8, Fig. 1) two of the 12 lines underwent endomixis. A third line was in endomixis in the seventeenth five-day period: almost simultaneously (in periods 16 and 18) two lines were in endomixis in the normal concentration, so that no special significance can be attributed to this.

Thus the change to the unfavorable high concentration apparently induced endomixis in 2 of the 12 lines.

At the fourteenth period a new group of 12 lines was branched off from the normal concentration to the concentration 100 times as great (100 Fl II, Fig. 1): it was continued through 4 periods. The unfavorable effects were shown about as before (Table I), but endomixis was not induced.

Restoration to normal concentration after culture at the high concentration:—In the case of Group I cultivated at the hundred-fold bacterial concentration, after seven five-day periods (at the 14th period of the experiment as a whole), a group of 12 lines was restored to the more favorable normal concentration. Fission rate was restored to normal, the proportion of deaths and of chains was greatly decreased (Table I). The normal size of the animals was restored after five days. Endomixis occurred in 3 of the lines in periods 17 and 18, but this cannot be attributed to the change to the normal concentration, since in the 100-fold concentration, as well as in the original culture in normal conditions, lines were in endomixis at practically the same times (in periods 16, 17, 18, Fig. 1).

Bacillus Niger

At the beginning of period 5, and again at the beginning of period 13, derivatives of the normal control group in *Flavobacterium* were washed and transferred to a medium containing *Bacillus niger* in place of *Flavobacterium* (see Fig. 1, " Niger I " and " Niger II "). The niger medium roughly corresponded in concentration to that of *Flavobacterium* (one 1-mm. loop to 20 cc. of culture fluid). In both cases there was a sharp lowering of the fission rate in the first period after transfer, but this was soon partially recovered, the average fission rate being 8.23 (Group I) and 8.91 (Group II) as compared with 10.38 in the entire *Flavobacterium* culture medium, so that these differences are hardly significant. As Table I shows, none of the other criteria indicate that *B. niger* is less favorable than *Flavobacterium*. Yet endomixis was at once induced in *B. niger*—in 7 lines in Group I, in 11 lines in Group II (Fig. 1). In all there were 23 lines in endomixis in Group I of *B. niger*, 18 in Group II—as compared with but 5 in the entire course of the *Flavobacterium* culture. The number of endomictic lines per 100

line-days was in *B. niger* a little over 3, as compared with 0.4 in *Flavobacterium* (Table I). In Group I, 9 lines underwent a second endomixis while in *B. niger,* and the interendomictic interval was short, ranging from 24 days (44 generations) to 48 days (79 generations), as compared with 75 to 103 days in *Flavobacterium.* Similar relations are found in Group II of *B. niger.*

It appears clear, therefore, that culture in *B. niger* greatly increases the frequency of endomixis, and that this is not a result of unfavorable conditions, for *B. niger* appears otherwise as favorable as *Flavobacterium.*

When derivatives from Group I in *B. niger* were restored to *Flavobacterium,* after 40 days in *B. niger,* there was after a few days a rise in fission rate. No endomixis occurred in those thus restored to the normal medium.

High Concentrations of B. niger

When individuals of Group II in *B. niger* were transferred to a concentration of *B. niger* 100 times as great as normal, there was no appreciable change in fission rate, mortality, or in number of imperfect divisions. Endomixis, however, was increased, 8 lines undergoing endomixis in the 4 periods of this culture (Fig. 1). This agrees with the fact previously noted, that the presence of *B. niger* in the culture medium increases the frequency of endomixis, and that this increase is not the result of the unfavorableness of *B. niger.*

Bacillus cereus

Two groups of 12 lines were transferred from *Flavobacterium* to a normal concentration of *B. cereus,* at periods 5 and 13 of the experiment (Fig. 1). Fission rate was very slightly decreased, and the animals were on the average a little smaller in size than in *Flavobacterium,* but mortality did not increase and there were no imperfect fissions, forming chains. Thus this bacillus can hardly be considered appreciably harmful. Yet, as in *B. niger,* endomixis was definitely increased in frequency. There were eleven endomixes in the 12 lines of Group I, 12 in those of Group II, while in the same periods the lines in *Flavobacterium* underwent but 4 endomixes. Seemingly *B. cereus* is less effective than *B. niger* in producing endomixis, since none of the lines in *B. cereus* underwent a second endomixis, while a number of those in *B. niger* did so, though the number of periods was the same for the two types of culture.

Restoration of lines of *B. cereus* culture to *Flavobacterium* did not result in the production of endomixis.

High Concentration of B. cereus

Derivatives of Group II in normal *B. cereus* were transferred at the nineteenth period to a concentration of *B. cereus* about 100 times as great (Fig. 1). This decreased the fission rate by about one-half, increased the mortality, and the formation of abnormal chains, and caused the production of large crystals in the cytoplasm. Conditions were clearly unfavorable, but endomixis was not induced.

Bacillus prodigiosus

At periods 5 and 13, two groups (I and II) were derived from the normal control cultures in *Flavobacterium*, and were cultivated in the normal concentration of *B. prodigiosus*. Fission was slightly lowered and the number of abnormal chains slightly increased (Table I); also a few individuals without macronuclei were observed. Thus the medium containing this organism must be considered slightly unfavorable. No increase in the frequency of endomixis was produced.

Bacillus coli

Groups I and II, derived in the usual way from the *Flavobacterium* cultures at the beginning of periods 9 and 13, showed little change in fission rate, but there was a considerable increase in the proportion of deaths, and of abnormal divisions (chains). Also numbers of individuals without macronuclei occurred. In Group I, four cases of typical endomixis occurred (Fig. 1). In addition, there occurred in most of the lines irregular fragmentation of the macronucleus. This began in both groups a few days after the transfer to *B. coli*, and continued for about 10 days. The fragments varied from day to day in size and number. They were scattered through the cytoplasm, or sometimes were in pockets of the macronucleus. The macronucleus was frequently present in two pieces; often it exhibited signs of activity, in the possession of projections of various sizes. Some of the stages closely resembled figures presented by Diller (1936, p. 37) for the process of hemixis in his types *B* and *C*. This fragmentation was in most cases not followed by the typical climax stage of endomixis, though it was in one of the lines. In other cases, after the disappearance of the fragments, the macronucleus remained normal. Such fragmentation occurred only in media containing *Bacillus coli*.

Summary

1. In the long-lived stock of Woodruff, cultivated in a standard medium containing a normal concentration of *Flavobacterium brunneum*,

endomixis occurred only at very long intervals, of 75 to 109 days, or more. Increase in the quantity of *Flavobacterium* to 100 times the normal concentration made the conditions very unfavorable, but hardly increased the incidence of endomixis.

2. Substitution of *Bacillus niger* for *Flavobacterium*, in the same proportions, brought on endomixis and increased its frequency, although it did not make the conditions unfavorable. Increasing by 100-fold the concentration of *B. niger* caused a further increase in the frequency of endomixis, but still without making the medium unfavorable.

3. *Bacillus cereus* was not appreciably unfavorable in its action on *Paramecium*, yet like *B. niger* it increased the frequency of endomixis. Its tendency to induce endomixis appears to be slightly less than that of *B. niger*. Increasing the concentration of *B. cereus* 100-fold made conditions very unfavorable, but did not increase the tendency to endomixis.

4. *Bacillus prodigiosus* is slightly unfavorable as compared with *Flavobacterium*, but it does not increase the frequency of endomixis.

5. *Bacillus coli* is unfavorable as compared with *Flavobacterium*. It produces a tendency to fragmentation of the macronucleus, usually not followed by complete endomixis.

6. Thus in these experiments there is no correlation between the unfavorable effect of certain bacteria and their tendency to produce endomixis. Certain species (*Bacillus niger* and *B. cereus*) are not unfavorable to *Paramecium aurelia* yet (in this stock at least) they much increase the frequency of endomixis. Certain other conditions that are unfavorable (particularly high concentrations of *Flavobacterium*) do not increase the frequency of endomixis.

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CONTRIBUTIONS TO THE STUDY OF DEVELOPMENT OF THE WING-PATTERN IN LEPIDOPTERA

WERNER BRAUN

(From the Department of Zoölogy, University of California, Berkeley, California)

Introduction

The mechanism of pattern determination in Lepidoptera wings has long been a favorite model for the study of gene action. Every study of developmental processes is concerned with the study of development of manifold structure, which finds its best object in the study of pattern, e.g. the pattern of a butterfly-wing. Goldschmidt tried the first successful analysis of the factors responsible for the development of this pattern and expressed it in his theory of different velocities of developmental processes. Although most parts of his analysis could be experimentally checked and extended by Kühn and his school, the decisive observations (" relief-stage ") of Goldschmidt's work were either not found, misinterpreted, or doubted by several investigators. An additional and more extended investigation of those stages was therefore necessary and will be furnished in this paper.[1]

Previous Experiments on the Development of the Wing-pattern

Goldschmidt's Investigations

In his book " Physiologische Theorie der Vererbung " (1927), Goldschmidt contributed an extensive analysis of the pattern problem.

A study of the development of the individual wing furnished the most decisive point in this analysis. Goldschmidt (1920, 1923) observed that the later wing-pattern is already completely represented in the young pupal wing, long before pigmentation takes place. It can be made visible by detaching unpigmented pupal wings from the body and allowing them to dry. After a short time a relief was visible on the former homogeneous-looking wing.

Later white scales became erected and formed the raised regions and later dark scales collapsed and formed the depressed regions. The whole relief represented the later wing-pattern in every detail. He observed this " relief-stage " in *Platysamia cecropia* L., *Lymantria, Thais*

[1] Acknowledgment for help and advice is due to Professor R. Goldschmidt and to the United States Government for assistance rendered through its Works Progress Administration Official Project No. 465-03-3-192.

and several other species. Goldschmidt assumed that in certain parts of the pattern the scales are still soft bags filled with blood, which collapse when dried out. In other districts, however, the scales are already chitinized and do not collapse when dried out. Because the later dark scales are consequently softer at this point of development than the later white scales, he concluded that later dark scales exhibit a slower development than later white scales. He arrived at the conclusion that the different velocities of development for the different parts of the wing were primarily responsible for the formation of wing-pattern.

According to his theory the different velocities of development start with the mosaic of the epidermis cells and their product, the scales which develop with different speed in different parts of the wing. A section through the young pupal wing in a certain stage would accordingly show us different parts of the wing in different stages of development.

At the same time different chemical substances will be present in the body, which if deposited in the scales will determine the color. These substances, like tyrosine, carotine etc., can be end-products of metabolic processes and a special production of them for the coloration-processes is not necessary. The deposition of these substances in the scales can be dependent on a certain colloidal stage of the chitin. If at a certain stage in the development tyrosine is present, it will only be deposited in those scales which represent a certain condition of the chitin at this moment, which means only a certain part of the pattern. Other parts of the pattern will react according to their stage of chitin-development and the pigmentation substances present. The development of a very complicated pattern can thus be explained by a very simple mechanism.

Goldschmidt (1920, 1927) then tries to trace these processes back in development and discusses the determination-points for these differences in developmental velocities ("sensitive period," determination-stream, Liesegang phenomenon).

The Investigations of Kühn and His Co-workers

Kühn and his co-workers tried to attack the same problems and furnished further experimental material. They worked with the flour-moth, which shows a pattern consisting of dark and light pattern-elements which are partly symmetrical (see Fig. 1). The various parts of the pattern do not only contain different amounts of pigment but the shape and size of the scales in various districts differ also. Therefore, the determination of the pattern is here not only a process of distributing different amounts of pigment but a morphogenetic process as well.

Feldotto (1933) determined the sensitive periods for this pattern and showed that heat-treatment at the pupal age of 12–72 hours was

able to modify the development of the different parts of the pattern. Each system of the pattern has its particular time of maximal modification at different stages of development.

Kühn and von Engelhardt (1933) cauterized the young pupal wing and thus were able to show that a determination-stream spreads over the wing from 48–60 hours of pupal age.

A period of particularly distributed cell divisions can be considered as the first reaction to the process of determination. These cell divisions were first described by Köhler (1932). He reported that from 36–84 hours of pupal age a great number of cell-divisions are visible which have their maximal distribution in districts of subsequent dark pattern elements while subsequent light pattern districts show few cell-divisions. The distribution of cell-divisions therefore resembles the subsequent pattern of pigmentation and Köhler consequently called it mitosis-pattern. A mitosis-pattern seems to be the predecessor to the later wing-pattern.

Braun (1936) examined the mitosis-pattern more closely with special regard to its rôle in the development of the wing-pattern.

He showed that this mitosis-pattern is visible from 36–148 hours of pupal age. Subsequent dark pattern-districts always represent maxima of mitotic divisions, while the minima are in later light pattern-districts during this period. The mitosis-pattern spreads in a wave-like fashion over the wing from the proximal base to the distal margin. Two such mitosis waves are distinguishable during the period of mitotic division. The first one goes over the wing from 36–84 hours, and represents cell-divisions of hypodermis-cells and the first division of the scale-building cells. The second wave starts at 84 hours and ends at 148 hours and represents the second division of the scale-building cells. When the first proximal divisions occur we find no mitosis in distal parts of the wing, with the exception of the cell-divisions of the later marginal scales, which start developing earlier and continue at a faster rate throughout the whole wing-development. Furthermore, it could be shown that this mitosis-pattern is the first sign of a completed determination of the wing-pattern.

Then an attempt was made to investigate the rôle of this mitosis-pattern in the development of the wing-pattern. It could be shown that the districts of mitosis-maxima (presumptive dark districts) represent districts of greater intensity of cell-division. Throughout the stages of development following the mitosis-period, later dark districts show a greater number of cells per unit than later white districts. The size of cells in the presumptive dark districts is accordingly smaller. This observation could be checked on the adult wing also, where one finds

more scale-building cells per unit in dark districts than in light districts. In addition it was found that all scales grow out simultaneously. From these observations it was concluded that a different intensity of cell-division was primarily responsible for the formation of the pattern. The principle of different velocities of development was discarded in favor of the principle of different intensity of cell-division as the primary factor in the development of a pattern. Köhler and Feldotto (1937) again tried to discard the existence of different developmental velocities for Lepidoptera in a recent paper. Their argument is based on their failure to find the scales of different districts in different stages of growth during experiments on scale-development. Furthermore, it had not been possible to observe the relief stage in *Ephestia.*

In response to these views expressed by the *Ephestia*-workers, Goldschmidt showed in recent publications (1938) that the observations made on *Ephestia* are in perfect harmony with his discoveries. The fact that we find more cell-divisions in certain districts during the mitosis-period means that the cells in these districts have not yet reached the end of their multiplication period. These districts are the later dark districts, they show a retarded development not only in the relief-stage but at this early stage as well. The cells in later white districts show less cell-divisions because they have reached the end of their multiplication period earlier and therefore differentiate faster than the later dark cells. (An investigation concerning the number of divisions which a cell undergoes in later dark or in later white districts would probably prove this explanation. Such an investigation is now under way.) The observation of the simultaneous growing out of scales does not interfere with this theory, because the different speed of differentiation of the scales in the different districts does not necessarily mean different growth. The process of growth might be nearly identical for all wing areas, while the different speed of development should be noticeable by differences in the histological structure of the scales, which would be hard to examine at this early stage.

To test these views it was necessary to undertake a more detailed investigation of the relief stage, where the different velocity of development for the different pattern districts was demonstrated so clearly. Furthermore, an attempt had to be made to find additional evidence for the different speed of development at stages previous to the relief stage. The relief-stage had to be found in *Ephestia,* where the mitosis-period had been found. Finally the mitosis-period had to be observed in other Lepidoptera, where the relief-stage had been demonstrated previously. The results of these investigations, which fully confirmed the validity of Goldschmidt's views, are described in the following section.

New Investigations on the Determination of the Wing-pattern

Material and Methods

Ephestia kühniella Zeller, *Platysamia cecropia* L. and *Papilio ajax* L. were the objects of the experiments to be described. *Ephestia* cultures were kept according to the method described by Kühn and Henke. *Cecropia* and *Papilio* pupae were obtained in the fall and stored in a refrigerator, from which they were put into an incubator at any time desired. They started development as soon as they were placed in a warmer temperature. All pupae developed at 25°. Fifty pupae of *Platysamia cecropia,* fifty pupae of *Papilio* and approximately one hundred and fifty pupae of *Ephestia* were used for these investigations.

The Appearance of the Relief Stage

As pointed out before, *Ephestia*-workers have never been able to find the stage in the pupal-development which is decisive for the theory of different developmental velocities, namely the relief stage. Therefore the first step was to hunt for this decisive stage in the development of the flour-moth. In order to become acquainted with the stage in question, the author repeated Goldschmidt's (1920) experiments on the *Cecropia* moth and *Papilio*. The observations confirmed Goldschmidt's results completely.

Platysamia cecropia L.—The wings were removed from the pupae at a time when no pigmentation had yet taken place, the best time being one to two days before pigmentation. They were then placed on a slide and allowed to dry. A relief appeared which showed subsequent white parts erect, subsequent dark parts collapsed. This stage is relatively short, approximately 3–5 per cent of the whole pupal stage, and was observed on ten *Cecropia* wings.

The relief of the *Cecropia*-wing is especially clear in the distal parts where the dark and yellow half-moon is located on the pigmented wing. (For excellent photos of this stage see Goldschmidt's 1920 paper.)

Papilio ajax L.—The same stage was easily found in *Papilio ajax*. Here too the relief-stage takes 3–5 per cent of the complete time of pupal development. It is very clear all over the wing, corresponding to the mature wing-pattern, and appears approximately twenty minutes after the wing has been put on a slide for drying. The subsequent light parts of the wing are indicated by clearly erected parts, the subsequent dark pigmented districts of the wing are all collapsed. This stage could be observed in fourteen wings which were removed from the pupae one to two days before pigmentation started. The different unpigmented pattern districts of the wings were visible even without drying. If the light

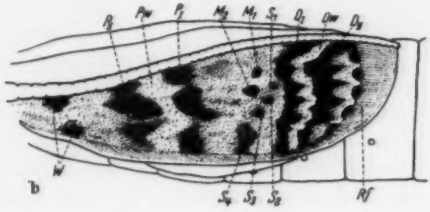
was shone in a certain way on the still attached and living wing, the districts of subsequent dark pigmentation and light pigmentation were clearly distinguishable by what appeared to be a difference of consistency of the areas in question. This difference can be demonstrated even more clearly, if one puts the wing into water. Again the districts of subsequent different pigmentation can be easily distinguished on the completely unpigmented wing. The action of two time factors for this period could be observed in *Papilio*. (1) At the moment pigmentation started, the relief disappeared in pigmented parts, while unpigmented parts showed the relief distinctly. (2) It is known that the hind wing always shows a faster development than the fore wing (e.g. pigmentation). Consequently the relief was already present on the hind wing, while all scales were still soft on the fore wing. The relief of the hind wing disappeared earlier because pigmentation set in earlier. (Pictures of the relief stage in species closely related to *Papilio* can be found in Goldschmidt's 1923 paper.)

Ephestia k.—The corresponding stage was found without much difficulty in *Ephestia* at eight to nine days of pupal age. That it had never been noticed before is surprising. The wing of the pupa was easily detached after the surrounding chitin was broken and carefully removed. Then the wing was placed on a slide and allowed to dry. After about fifteen minutes the two later dark bands appeared clearly as collapsed parts on the dried wing (Figs. 1 and 2). The action of an additional time-factor could be observed on these wings. They showed that the relief-stage proceeds like many other processes in the development of the flour-moth (mitosis-pattern, pigmentation) in a wave-like fashion from the proximal base to the distal margin of the wing. In early stages the relief of the proximal band only was visible, while in distal parts all scales were collapsed (Fig. 2 *a*). A little later the whole wing showed the relief, scales both of the proximal and distal bands being collapsed (Fig. 2 *b*). The marginal scales were an exception. They always exhibited a faster development, first noticeable during the mitosis period, and they were already erected in distal " white " parts in the early relief-stage. As in *Papilio*, the districts of subsequent different pigmentation were visible on the undried wing if the light was reflected in a certain way, as well as when the wing was put into water. These observations were based on a study of approximately sixty pupae.

The Chitinization of Scales during Pupal-development as Proof of the Different Velocity of Development

Sections.—The fact that no regional differences in the speed of outgrowing scales could be detected has been mentioned before (Köhler,

1932; Braun, 1936; Köhler and Feldotto, 1937). It was necessary to make sections of the wings in the relief stage to see if erect scales show a morphological difference at this stage. Such sections were made from



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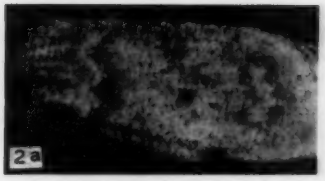

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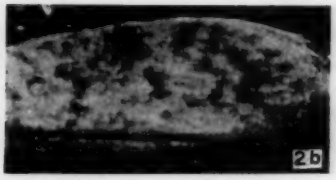

2b

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FIG. 1. Pigmented pupal wing of *Ephestia kühniella*. P_I, P_{II} proximal dark bands, D_I, D_{II} distal dark bands, M_I, M_{II} middle-spots.

FIG. 2*a–b*. Relief-stage of the pupal wing of *Ephestia kühniella*. *a*, early relief-stage showing the proximal band clearly, *b*, later relief-stage showing proximal band, distal band and five marginal spots. × 9.

FIG. 3. *Papilio ajax*. × ⅔.

eight *Cecropia* wings (through the district of the "half-moon"), from twelve *Ephestia* wings, and six *Papilio* wings, but no morphological differences in the stage of development of collapsed or erect scales of different forms were noticed. In many Lepidoptera white and dark areas are composed of scales of different forms, correspondingly later dark parts showed larger scales on the *Cecropia* wing than later white parts.

Consequently the different speed of differentiation of the scales in the different pattern districts does not manifest itself by different stages of growth. Growth seems to be the same for all parts of the wing. The difference therefore has to be sought in different velocities of the chitinization process of the later white and dark scales at this stage as suggested by the soft condition of later dark areas.

Chemical Tests.—In fact, such a difference could be detected by chemical reactions. There is not much known about the chemistry of chitin, but P. Schulze (1922) described a differential reaction for hard and soft chitin. The object is first saturated with iodine for a short time, blotted with filter paper, and finally covered with a solution of $ZnCl_2$. The reaction will show very soon but disappears after some time. Hard chitin will show a violet-brown color, soft chitin, a light brown. Ten unpigmented *Cecropia* wings were treated accordingly and showed a distinct reaction. Subsequent white parts appeared dark; subsequent dark parts appeared light. This experiment exhibits clearly that the different parts are found in different stages of chitinization. The same experiment was repeated with six pupae of *Papilio* and twenty pupae of *Ephestia* and always resulted in a clear pattern negative. Even the sections which showed no morphological difference reacted distinctly to this chitin test. The chitin reaction worked during the whole relief stage. It would have been desirable to check this test by making the same experiment with an adult pigmented wing. In this case all scales should show the violet-brown reaction. However, it was not possible to extract the pigment from an adult wing, which would have been necessary in order to obtain a bleached wing on which the reaction would be visible. Several other chitin reactions were tested, but none of them gave as clear a result as did the iodine-zinc reaction.

Test with Polarized Light.—After it had been proven that the different speed of development actually can be shown to be the different velocity of chitin hardening, an experiment to test the refraction of the differently chitinized scales was tried. Adult scales are doubly refracting, and it was hoped that one could find a stage where one kind of scales was singly refracting. Scales from different regions were investigated under crossed Nicols, but no clear results were obtained. It was men-

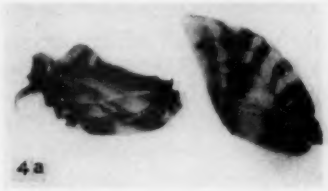
tioned before that the scales from different regions have different forms, and this difference may add to different refraction effects.

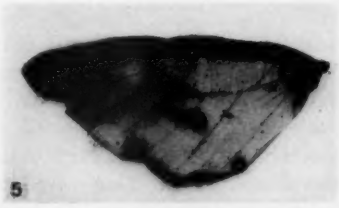
Tyrosine Reactions.—The next experiment was an attempt to produce premature pigmentation artificially in order to watch the reaction of the different regions. Studies on the chemistry of melanin formation by several authors have shown that two components are necessary for pigmentation: a chromogen and an oxydase. Oxydase (e.g. tyrosinase) is always present in the blood of Lepidoptera as may be easily shown, while the chromogen is supposed to enter the wing at a definite stage of development (the time of pigmentation). Oxydase and oxygen together produce a melanin pigment. If the blood of Lepidoptera contains tyrosinase it should be possible to produce a dark pigment on the yet unpigmented wing by providing the necessary chromogen, e.g. by soaking the unpigmented wing in a solution of tyrosine.

(1) *Papilio ajax.*—An unpigmented pupal wing of *Papilio* at the time of the relief stage was put in a saturated solution of tyrosine. At the stage in question the pupal wing is always folded in a definite manner, which allows for the subsequent expansion of the wing after hatching. The wing is compressed in length and width to one-third of its actual size. This results in a wave-like appearance of the epithelium, with the scale-bearing epithelium as the crest and the epithelium without scales as the trough. (These folds do not interfere with the observation of the relief-stage on the pupal wing.) After immersion in the solution for four hours at 30°, the wing exhibited the following characteristics: it was completely stretched like the wing of the imago and showed the complete pattern of an adult pigmented wing; subsequent dark parts were dark, subsequent light parts were white (unpigmented) (Figs. 3 and 4). Microscopical examination of wings which had been treated with tyrosine revealed that the pigment is actually deposited in the scales. It is, however, not as black as the normally deposited pigment in the wing of the hatched butterfly. This experiment shows that a certain viscosity of the chitin is necessary for the deposition of a pigment in colloidal solution. After a scale has reached a certain point of hardening it cannot deposit any more pigment. The tyrosine can react with the tyrosinase in the still soft scales of subsequent dark parts, but no reaction can take place in the hardened scales of subsequent light parts. The tyrosine experiment demonstrates clearly how the different speed of hardening in different regions leads to the pattern of pigmentation.

Pupal wings of different age were treated accordingly to investigate the length of the stage during which a tyrosine reaction is possible. Very young and soft pupal wings, those on which the scales have just started to grow, show after treatment with a tyrosine solution a light

gray color all over the wing with very faded contrasts of dark and light areas. The veins, however, and the marginal scales show a dark black pigment (Fig. 5). A control wing, dried at the same stage, shows all scales collapsed. In a later stage a dried-out wing will still show all scales collapsed, but if it is treated with tyrosine, a pale pattern will become visible, and subsequent dark parts will show black reaction, while subsequent light parts will not show any reaction with tyrosine (Fig. 6). With the help of the tyrosine reaction we thus can trace back the differential velocity of chitinization to a very early point in the scale de-





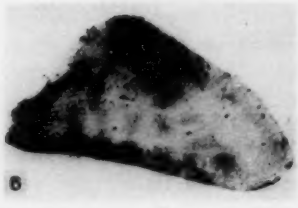


FIG. 4*a–b*. Pupal wings of *Papilio ajax* after treatment with tyrosine ca. 1 day before pigmentation begins to set in. *a*, fore wing and hind wing × 1½, *b*, part of the fore wing × 2½.

FIG. 5. Pupal wing of *Papilio ajax* after treatment with tyrosine ca. 2 days before pigmentation begins to set in. × 2½.

FIG. 6. Pupal wing of *Papilio ajax* after treatment with tyrosine ca. 3 days before pigmentation begins to set in. × 2½.

velopment. Long before the relief stage we are able to produce the later pattern, which means that before we can see the roughly morphological difference of the chitinization in the relief stage, we are able to demonstrate it by chemical means. The chitin of subsequent white scales either hardens earlier and does not allow the tyrosine to penetrate, or at these moments the white scales do not contain sufficient blood which, as carrier of the oxydase, is necessary for the reaction with the tyrosine. The first explanation seems more plausible. All the wings which showed the tyrosine reaction still have to be examined microscopically more carefully to complete our knowledge of the details of this process. But

the fact remains that from a very early stage, the different velocities of the different districts can be shown long before we can observe collapsed or erected scales after exposure of the wing to air. The tyrosine reaction can be performed until normal pigmentation sets in on the wing and always gives a clear positive of the pattern. Eighteen wings of *Papilio* were used for these observations and *Papilio ajax* has proved to be an excellent object for these studies.

(2) *Ephestia k.*—The tyrosine reaction was tested on thirty flour-moth wings but did not show the bands distinctly, probably because the parts of the pattern of the *Ephestia* wing are not clear and limited enough.

It has been mentioned above that the pupal wings which are folded when removed from the pupae will always unfold after they have been placed in tyrosine. No other solution tested produced this phenomenon. Pupal wings from several species, among others pupal wings of *Drosophila*, were put in a tyrosine solution, and they always unfolded after one or two hours. This fact might be of great help for other embryological work in insects.

Dissolution of the Scales.—The next test was based on the following consideration. The scales show a distinctly different chitinization at the relief stage. It is easy to remove scales from the collapsed or erect parts. If the scales from both parts could be dissolved, the harder scales should need a longer time for dissolving. Mature scales on the wing of a hatched butterfly should show the same degree of chitinization in all parts, and white and dark scales should need the same time for dissolution. Such an experiment could furnish another proof that the different chitinization during development is really an expression of the differential speed of some developmental processes. All our experiments have a greater meaning if it can be proved that all scales are chitinized equally on the adult wing.

The experiments proved that this idea was right.

Papilio ajax scales from subsequent light and dark parts were dissolved in concentrated sulfuric acid. Subsequent dark scales start to dissolve as soon as touched by the acid. Subsequent white scales first show air bubbles when put into H_2SO_4, and then start to dissolve slowly (ca. 12 minutes). If the same experiment is repeated with scales from a mature pigmented wing, the white scales will show the same reaction as they did during the relief stage. This means they were already mature at this age. Dark pigmented scales, however, now show a longer resistance to the acid and start to dissolve only after hours of treatment. The prolonged time of the dark scales as compared with that of the white scales is probably due to the pigment deposition in the dark scales.

Scales from the same wing which were utilized for the observation of the relief-stage were used for this experiment. The same results were obtained in fifteen successive tests.

The Mitosis-pattern in Cecropia and Papilio ajax

In order to construct a general picture of the elements important for the pattern formation it was finally necessary to show that all the stages discussed (mitosis-pattern, different chitinization, relief-stage) are of general occurrence. Only the general presence of all stages, in particular relief-stage and mitosis-period, allows us to correlate them. The mitosis-pattern of *Ephestia* has been clearly demonstrated and closely investigated previously (Braun, 1936). The same kind of mitosis-pattern has been found now in ten wings of young *Cecropia* pupae, and follows the same principles as in *Ephestia*.

(The youngest *Papilio* pupae which we had in our laboratory were already too old to show the period of cell division.)

These observations confirmed that the mitosis-pattern, as well as the relief stage, is a general stage in the development of the wings of the Lepidoptera.

Discussion

The new evidence gained by these experiments confirms Goldschmidt's views completely. The tyrosine reaction, the relief stage and its chitin reactions, and finally the test of dissolving scales, leave no doubt that we are dealing with different velocities of development for the different parts of the pattern. This difference turned out to be a difference in the hardening or maturing of chitin. Under these circumstances it is only logical to interpret the mitosis-pattern in these terms too. We have seen that all the stages of development for different parts of the pattern are generally present in such different species of Lepidoptera as *Ephestia, Papilio ajax* and the *Cecropia* moth. *Ephestia*-workers neglected Goldschmidt's views on the basis of not finding different districts of the pattern in different stages of development, but they only considered growth, which does not seem to be an exact indicator for the different velocities. The indicator for these velocities seems to be of a purely histological nature. We have been able to demonstrate such an indicator in the process of chitinization of the scales. In interpreting the mitosis-pattern in the same terms as the subsequent stages of development we are aided by the similarity of time factors in both stages. The mitosis-period and the relief stage proceed wave-like proximally to distally over the wing. In both periods we find the mar-

ginal scales advanced in development as compared with all the other scales.

Based on former investigations and our new experiments we can describe the development of the pattern in Lepidoptera as follows:

In the very young pupal wing, districts of different visible as well as physico-chemical structure are present, partly produced by the localization of tracheas, veins and different surface conditions, perhaps also by processes in which a Liesegang phenomenon is involved. At a certain time in development a determination stream (or more than one) spreads over the wing and is distributed pattern-like according to the chemico-physical conditions present in the substrate and dependent upon the points of origin and directions of the stream or streams. In the case of *Ephestia* it can be shown that the subsequent dark districts are determined in the areas where the determination-stream stops. At any time before the occurrence of the determination-stream the subsequent pattern can be altered by extreme environmental conditions like heat treatment. Different parts of the pattern can be altered at definite limited periods, the " sensitive periods." Different phenotypes or genotypes are produced by the change of time-action of the determination-stream. The determination-stream determines districts with different speeds of differentiation. These different velocities of development are first visible in the mitosis-period which follows the period of the determination-stream. During this mitosis-period more cell-divisions are visible in later dark districts than in subsequent white districts, thus forming a mitosis-pattern, equal to the later pattern of pigmentation. The cells of the subsequent dark parts, which show a more intensive division process, have not yet reached the end of their multiplication period, in contrast to the subsequent white parts which enter their period of differentiation first. The mitosis-pattern spreads over the wing in the form of a mitosis-wave from the proximal base to the distal margin of the wing. Two such mitosis-waves can be observed. The first one is composed of cell-divisions of the hypodermis-cells and the first divisions of the scale-building cells. The second one is composed of the second cell-divisions of the scale-building cells. The different velocity of development for the different parts of the pattern can be observed again some time before pigmentation sets in. It can be made visible by the tyrosine reaction, the relief stage and its chitin reactions, and by the test of dissolving scales. These tests show that the subsequent dark parts, which have already been retarded during the mitosis-period, develop slower up to the time of pigmentation. The velocity of hardening of the chitin is different for the different parts of the pattern, and this difference finally leads to the pattern of pigmentation. Since a pigment is de-

posited in the chitin in colloidal solution, it can only be deposited as long as a scale has not reached a certain point of hardening. At a certain time of development pigment is present in the body and the subsequent dark parts, being still soft at this time, will deposit pigment; the subsequent white scales on account of their faster development, which has led to a harder chitinization, cannot deposit any pigment at this point. In this way the different velocities of differentiation of different areas of the wing throughout the pupal development starting from the time of the determination stream will lead to the pattern of pigmentation.

Summary

1. Goldschmidt's observations and ideas on the problem of different velocities of development for different parts of a pattern are described. The experiments of Kühn and his school and their negative views in regard to the idea of different velocities of developmental processes are summarized.

2. New observations confirm the existence of the "relief-stage" in the pupal wing of *Platysamia cecropia* and *Papilio ajax*, as well as in *Ephestia k.*, where it has not been previously observed.

3. It was observed that the relief-stage proceeds over the pupal wing from the proximal base to the distal margin in a wave-like fashion as is the case with the mitosis-pattern. The relief stage and the mitosis-pattern appear on the hind wing earlier than on the front wing.

4. No difference in growth of the scales was observed in sections through the wing during the relief-stage.

5. With the help of a chitin-reaction it could be shown that during the relief-stage the subsequent light parts of the wing were more chitinized than the subsequent dark parts.

6. A test with polarized light during the relief-stage gave no clear results.

7. With the help of artificial pigmentation (tyrosine-reaction) a complete pattern can be produced on the still unpigmented wing long before the relief-stage could be observed.

8. This tyrosine-reaction shows that a certain condition of the chitin is necessary to deposit pigment in the scales. Subsequent light parts of the wing are more chitinized at the time of pigmentation than subsequent dark parts and therefore cannot deposit any pigment. This experiment proves that the different velocities of development of the different parts of the pattern lead to the pattern of pigmentation.

9. By dissolving scales from subsequent dark and light districts in H_2SO_4 during the relief-stage and dark and light scales on the mature

wing, it could be actually shown that the scales are differently chitinized at the time of pigmentation and equally chitinized on the mature wing.

10. The similarity of processes during the mitosis-period and the relief stage and their general appearance in different species of Lepidoptera allow us to correlate them. Both stages express the different velocities of developmental processes for different parts of the wing, which finally produce the pattern of pigmentation.

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THE RELATIONSHIP BETWEEN THE PITUITARY GLAND AND THE GONADS IN FUNDULUS

SAMUEL A. MATTHEWS

(From the Thompson Biological Laboratory, Williams College, and the Marine Biological Laboratory, Woods Hole)

Introduction

From the extensive work on mammals, birds, reptiles and amphibians it is clear that in these forms the anterior lobe of the pituitary gland is an essential factor in the control of the sex cycle. In teleost fishes, however, there is little evidence concerning the rôle of the pituitary body. Houssay (1930, 1931) found that the injection of saline suspensions of the hypophyses from large fish (*Micropogon opercularis*) into small ones (*Cnesterodon*) was followed by the expulsion of eggs in from one to three days, although saline suspensions of muscle or saline solution alone produced no effect in control animals. This expulsion of eggs, however, preceded normal spawning by only 15 days. Cardoso (1934) injected saline suspensions of the pituitary bodies of *Pimelodus clarias* into other individuals of the same species. In both sexes he noted an increase in weight of the gonads over those of control animals, an effect much greater in the female. Pereira and Cardoso (1934), working with *Prochilodus,* injected saline suspensions of pituitary glands of the adult into females. In all cases ovulation occurred, varying from 24 to 96 hours after injection, although the injection of suspensions of nervous tissue into control animals did not result in ovulation. More recently von Ihering (1935) repeated these experiments, injecting suspensions of the pituitary glands of *Hoplius malabaricus* into two species of *Astyanax*. Mating activity was stimulated with an increase in size of both ovary and eggs over the untreated controls. When injections were made into *Prochilodus* of both sexes eggs and spermatozoa were obtained. Pituitary-like substances such as prolan and extracts of the pituitary gland have likewise been used in fishes with some success. Calvet (1932) placed young *Petromyzon* in aquaria containing pregnancy urine and found that the ovary increased markedly in size over that of the controls. Damas (1933) injected 30 lampreys with pregnancy urine and obtained expulsion of eggs in all cases. He also employed extracts of the pars anterior of the pituitary gland with similar results. Boucher,

Boucher and Fontaine (1934) injected the urine of pregnancy into eels and reported enlargement of the gonads in both sexes and evidence of the maturation of spermatozoa in the testis. On the other hand, wholly negative results were obtained by Koch and Scheuring (1936) after injecting *Phoxinus* with both prolan and an extract of the anterior lobe of the pituitary gland.

Effect of Injection of Pituitary Extracts

In an effort to determine whether or not the pituitary gland of *Fundulus heteroclitus* is concerned in the control of the sex cycle two methods were employed, the injection of pituitary glands or extracts of them, and extirpation of the pituitary body. The results of the injection method have been disappointing. In male individuals no effect which can be ascribed to the material injected has been observed. The following is a brief summary of the results of several experiments. Following the successful use by Burns and Buyse (1933) of extracts of mammalian pituitary glands in stimulating precocious sexual development in *Amblystoma,* injections of a crude, alkaline extract of the whole pituitary gland of sheep were made into the body cavity of *Fundulus.*[1] In the first series records were obtained from 28 males so injected, with a number of uninjected controls. While spermatozoa were obtained in a number of cases they were also obtained in control animals, developing in both groups after the individuals had been maintained in the laboratory for some time. This appearance of spermatozoa was possibly a result of the change from the lower temperature of the Delaware river in which they were collected to the higher temperature of the laboratory. At any rate, that the appearance of spermatozoa in these males was not a result of the injections was shown by further experiments. Twenty-four males were divided, 12 placed in an aquarium in a constant temperature room at 5° C. and the other 12 kept in an aquarium in which the temperature of the water varied from 18° to 22° C. with an average temperature during the experiment of 20°. Since the constant temperature room was illuminated by daylight the light conditions in the two rooms were fairly comparable. Half the animals in each room were injected daily with 0.1 cc. of sheep pituitary extract. Records were obtained on 18 of these animals. In the warm room the testes were activated and spermatozoa appeared in both uninjected and injected animals in all cases after 7 days. In the cold room no spermatozoa appeared in either injected or in control animals. Microscopic examination of the testes showed that those of the injected animals were comparable to those of the uninjected controls, those of both groups in the

[1] I wish to express my thanks to Dr. Oliver Kamm of the Research Laboratories of Parke, Davis and Company for this material.

cold room showing less activity than was the case in the testes of animals from the warm room.

In females a more varied injection program was employed in an attempt to produce ripe eggs. The following materials were injected: whole pituitary extract of sheep, Antuitrin S (Parke-Davis), extracts of fish pituitary glands (*Fundulus* and *Mustelus*), and freshly ground pituitary glands of *Fundulus*. Records were obtained from 35 injected females. Only 4 of these, animals injected in March and early April, delivered ripe eggs. The earliest case of this occurred on March 16 after the animal had been in the laboratory for 11 days and had received 3 injections of 0.15 cc. each of sheep pituitary extract. The other 3 cases delivered eggs late in April (April 20–28) about one month before eggs were obtained from normal animals (May 27). While these 4 cases suggest the possibility that injection of pituitary extracts into *Fundulus* in early spring after the ovocytes have grown to a certain size may cause them to mature more rapidly than they normally do, certainly the number of cases is too small to be of much significance. In the 31 other cases no ripe eggs were obtained and microscopic examination of the ovaries showed no significant differences between them and those of control animals.

Effect of Hypophysectomy

The second method employed to study the influence of the pituitary gland on the gonad cycle in *Fundulus* was the removal of the gland. The operation was performed essentially in the manner previously described (Matthews, 1933) but with several modifications. A single, short mid-ventral incision was made in the branchiostegal membrane, reaching anteriorly as far as the base of the tongue. Through this incision the tongue was dissected free from the tissues of the floor of the mouth. The tip of the tongue was then pulled backwards and held at right angles to the ventral surface of the head with the left hand, its wedge shape serving to hold the wound open. After cutting through the mucous membrane in the roof of the mouth the parasphenoid bone was cut through on three sides, bent to one side of the median line to expose the hypophysis, and then replaced after the pituitary body had been removed. If the original incision in the branchiostegal membrane was of the proper length the tongue could be tucked through the incision and the free tip would hold it in place, thus closing the wound in the branchiostegal membrane without sutures. Healing in these cases was much more satisfactory than in the earlier operations.

This operation was carried out at two periods of the year. One group of animals was operated on in March and April, the other in October, November and December. The mortality, as usual, was high.

Of 176 operated animals weight records and fixed gonads were obtained from only 73,—33 males and 40 females. When the gonads from these animals were examined differences were noted between them and

TABLE I

Percentage of total body weight formed by the testis in hypophysectomized Fundulus as compared with normal and operated controls

Normal and Operated Controls			Hypophysectomized Animals		
Date Killed	Days Post-Operative	GW/BW (per cent)	Date Killed	Days Post-Operative	GW/BW (per cent)
NOR Oct. 10.		0.22	Oct. 19	10	0.21
Oct. 10.		0.31	Oct. 19	13	0.19
Oct. 10.		0.27	Oct. 26	13	0.22
			Oct. 24	18	0.26
			Oct. 26	20	0.19
			Oct. 28	21	0.25
HYC Nov. 17.	39	0.76	Nov. 7	32	0.21
			Nov. 27	48	0.37
HYC Dec. 3.	53	0.71	Dec. 3	51	0.33
HYC Dec. 3.	56	0.49	Dec. 17	66	0.27
			Dec. 24	73	0.43
HYC Mar. 12.	12	0.46	Mar. 7	5	0.46
			Apr. 20	5	1.05
			Mar. 11	9	0.77
HYC May 5.	206	1.20	May 4	10	1.48
HYC May 5.	206	2.01	May 6	12	0.81
			May 6	12	1.62
			May 6	13	0.43
			May 8	14	0.24
			May 10	16	0.38
			May 10	16	0.54
			May 12	18	0.29
			May 13	19	0.44
			May 7	22	0.67
			May 14	29	0.41
			May 16	31	0.21
HYC June 11.	48	1.26	June 11	48	0.30
NOR June 11.		1.96	June 11	49	0.28
			June 11	55	0.17
			June 11	55	0.08
			June 11	57	0.28
			June 11	57	0.17
			May 5	206	0.15

those of controls, particularly in the males. These changes were apparent in the weight of the testis relative to the total body weight, and in both gross and microscopic appearance.

Table I summarizes the way in which the testis weight changes after

hypophysectomy. As has been shown (Matthews, 1938) during the normal seasonal cycle the testis forms the smallest percentage of the body weight from September to December and the greatest in May and June. When the pituitary gland is removed in October there is no significant difference in the weight of the testes of experimental animals as compared with those of controls even after 73 days. In the one animal which survived until the next breeding season, however (206 days), the difference in the proportion of the body weight formed by the testis as compared with that of control animals was striking. Obviously the testis in this animal had not enlarged with the onset of the breeding season as did that of the control. In the series from which the pituitary gland was removed in March and April and the animals killed during the next breeding season in May when the testis normally reaches its greatest size, the same difference between the weight of the testes of control and of hypophysectomized animals was noted, though in these cases they had been hypophysectomized only 48 to 57 days previously. How soon after operation this difference in weight is noticeable cannot be accurately determined from this series due to an insufficient number of animals killed early in the experiment. It is clear, however, that there is no difference up to about 10–12 days after removing the hypophysis. After 13 or 14 days, however, the proportion of the total body weight formed by the testis is consistently and notably less than in control animals.

In gross appearance the differences between the testes of hypophysectomized and control animals are clear from Figs. 1 and 2. In place of the large whitish testis of the control animal that of the hypophysectomized individual is smaller and has the gray, translucent appearance of the testes of sexually inactive animals killed in late fall.

The microscopic appearance of the testes of these animals also shows differences between control and hypophysectomized individuals. Although the testes of animals operated on in October show no significant weight differences as compared with those of controls, the microscopic structure of these testes is different. The testes of control animals killed in October show a large number of primary spermatogonia, a number of secondary cells, including both secondary spermatogonia and primary and secondary spermatocytes which are multiplying to form cortical cysts, as well as a number of spermatids and a few spermatozoa (Fig. 3). In two animals killed during mid-October from which the pituitary glands had been removed 10 and 13 days previously the primary spermatogonia are as numerous as in the controls and are dividing in both cases as indicated by the presence of mitotic figures. The striking differences between these two testes and those of control animals

killed at the same time lie in the reduced number of cells of the second type and in the small number of mitotic figures in these as compared with those in the control testes (Fig. 4). In fact, the cysts, which are prominent in the control testes, are small and poorly defined in those from the hypophysectomized individuals. Moreover, spermatids and spermatozoa are extremely scanty. The testes of these two hypophysectomized animals present the picture of multiplication of spermatogonia without subsequent maturation of sperm cells. If this lack of maturation of spermatozoa is due to the lack of the pituitary gland, then it might be expected that such differences would be more marked in those hypophysectomized animals allowed to live on into the normal breeding season when maturation of sperm cells is most rapid. That such is the case is indicated in a number of such cases. Obviously the removal of the pituitary gland must precede the killing of the animal by a sufficient number of days for the effect on the testis to be noted. Thus in none of the animals hypophysectomized in March and April and killed from five to twenty-two days later are any differences observable in the testes of the operated and control animals. In fact, one of these animals killed on April 20, five days after removal of the pituitary gland, showed a definitely active testis (Fig. 5). The differences between this testis and that of one of the same series of operated animals killed on May 16 are well marked (Fig. 6). In the latter case the only cells present are primary spermatogonia and a few spermatids and spermatozoa. The differences between the two are such as might be explained by assuming that on April 15, when the pituitary glands were removed, rapid multiplication and maturation of the spermatogenic cells were well under way in preparation for the next breeding season. In the animal killed five days after removal of the hypophysis the lack of the gland had not as yet produced any effect on the testis. Twenty-six

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EXPLANATION OF PLATE I

FIG. 1. Testis of control animal which had its pituitary gland exposed but not removed on October 10 and was killed May 5. × 1.4.

FIG. 2. Testis of animal hypophysectomized on April 15 and killed June 11. × 1.4.

FIG. 3. Cross-section of testis of normal animal killed October 10. × 194. *C*, cortical cyst containing spermatocytes; *PS*, primary spermatogonia; *S*, spermatids.

FIG. 4. Cross-section of testis of animal hypophysectomized October 9 and killed October 19. × 194. *PS*, primary spermatogonia; *M*, medulla of testis.

FIG. 5. Cross-section of testis of animal hypophysectomized April 15 and killed April 20. × 80. *C*, cortical cysts containing cells which are dividing rapidly; *SZ*, spermatozoa.

FIG. 6. Cross-section of testis of animal hypophysectomized April 15 and killed May 16. × 80. *PS*, primary spermatogonia; *SZ*, spermatozoa.

1

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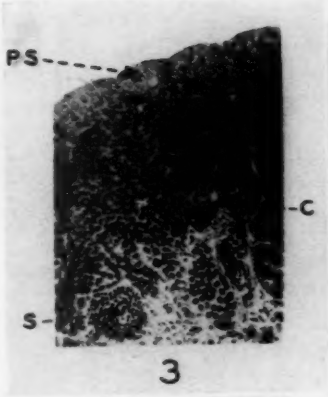

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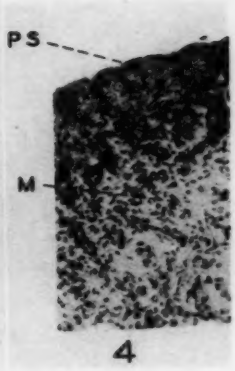

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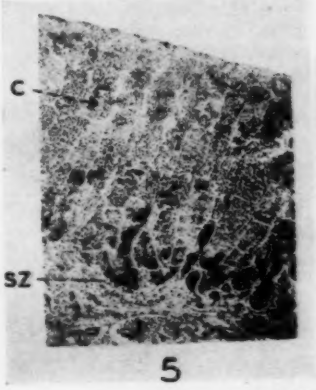

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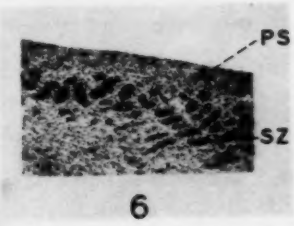

6

PLATE I

days later, however, only a few spermatids and spermatozoa remain from the mass that had already been produced. Evidently after the lack of the pituitary gland has had time to manifest itself, further maturation of spermatogenic cells is either very much retarded or perhaps altogether lacking.

The most striking examples of this change in the testis following hypophysectomy were found in those animals which were allowed to run well over thirty days after removal of the pituitary gland and then killed close to or during the breeding season. Seven of these cases were observed with 5 control animals killed at the same time. These animals were killed from 48 to 206 days after removing the hypophysis.

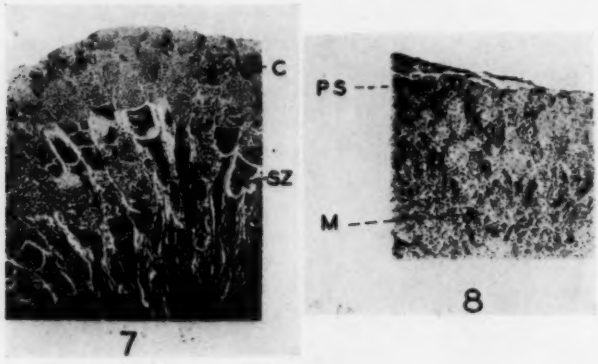

PLATE II

FIG. 7. Cross-section of testis of control animal which had its pituitary gland exposed but not removed on April 24 and was killed June 11. × 80. *C*, cortical cysts with cells which are dividing rapidly; *SZ*, spermatozoa.

FIG. 8. Cross-section of testis of animal hypophysectomized April 24 and killed June 11. × 80. *PS*, primary spermatogonia; *M*, medulla of testis.

With the exception of minor individual variations, differences in the microscopic appearance of the testes of these animals as compared with those of controls are much the same as those already described. The testis of all the hypophysectomized animals show only primary spermatogonia, a few scattered secondary spermatogonia and either very few spermatids and spermatozoa or none at all (Figs. 7 and 8). It might be noted, however, that in all these animals the primary spermatogonia are dividing, even in the one case from which the pituitary gland had been removed 206 days previously.

The data for hypophysectomized females are at present very scanty. As in the male, there is little change in weight of the female gonad in

those cases killed in the fall months and differences in the microscopic structure of the ovary of hypophysectomized as compared with control animals are hard to detect. Although more females were operated on than males the mortality of these animals was particularly high as the breeding season approached. As a result records on hypophysectomized females killed during May and June have been obtained on only a few animals. The oldest of these in post-operative age was killed only 55 days after removal of the hypophysis and records were obtained on only two others which were killed more than 30 days following hypophysectomy. In all three cases, however, the ovary was lighter relative to the total body weight than in control animals, several of which ran 206 days after exposing but not removing the pituitary gland. An example is provided in case *HY* 4/17–6/11 in which the ovary formed 1.52 per cent of the body weight 55 days after removing the hypophysis as compared with *HYC* 10/10–5/5 (206 days post-operative, 4 individuals) in which the average weight of the ovary constituted 2.87 per cent of the body weight. It is obvious, however, that more cases from which the hypophysis has been removed for more than 30 days prior to the normal breeding season must be obtained before the effect of the loss of the pituitary gland on the ovary may be accurately determined.

Summary

Injections of pituitary extracts into male *Fundulus* have been practically without stimulating effect on the testis. In females records were obtained from thirty-five cases of which only four delivered eggs earlier than did control animals, one case delivering ripe eggs six weeks, the other three cases about four weeks before the normal breeding season.

After removing the pituitary gland the gonads undergo regressive changes as compared with the controls, changes which are particularly striking in the testis. When the pituitary gland was removed in the fall and the animals were allowed to run until the next breeding season in May and June the testes failed to enlarge as they normally do. Microscopically such testes showed numerous primary spermatogonia but very few spermatocytes and practically no spermatozoa. When the pituitary gland was removed in March and April, after the testis had already enlarged somewhat, fourteen to twenty-one days after operation the testes had become notably smaller than in control animals, and showed only primary spermatogonia with a few spermatids and spermatozoa. These results indicate that the pituitary gland exerts a controlling influence on the seasonal cycle which the testis of this teleost fish exhibits. This influence is apparently of greater importance in maturation than in proliferation of the germ cells.

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STUDIES ON VIRUS DISEASES OF FISH

II. Lymphocystis Disease of Fundulus heteroclitus [1]

RICHARD WEISSENBERG [2]

(From the Effingham B. Morris Biological Farm of the Wistar Institute, Bristol, Pa.)

At the meeting of the American Society of Zoologists at Indianapolis, December 1937, the author presented a paper and a demonstration concerning " intracellular parasitism in fish producing a gigantic growth of the infected cells." [3] This paper also dealt with parasitic protozoa as well as with the lymphocystis virus disease and the peculiar structure of the lymphocystis cells. With reference to this latter, Dr. Roland Walker, Biological Laboratories, Rensselaer Polytechnic Institute, Troy, New York, sent to the author sections through a tumor of the common salt water killifish *Fundulus heteroclitus,* which he had preserved in August, 1937, at the Marine Biological Laboratory, Woods Hole, supposing that this tumor represented something similar to the demonstrated hypertrophied fish cells.

Studying these preparations I could easily verify that the tumor indeed represents a lymphocystis tumor, never described hitherto on *Fundulus hetroclitus.* I am greatly indebted to Dr. Walker, who decided to leave the description of this interesting observation entirely to me.

The significance of this discovery is not represented so much by the finding of a new host of lymphocystis disease, but by the fact that this fish is known to be easily kept under laboratory conditions. Therefore, it can be hoped that lymphocystis disease could be cultivated in the laboratory for further experimental studies if sufficient attention were paid to a new appearance of this virus disease on *Fundulus heteroclitus.*

The writer estimates from the series of sections that the tumor probably had a diameter of 2 to 3 mm. The localization of the tumor was in the membrane of the tail fin. The preserved fish was the only specimen among 200 *Fundulus heteroclitus* on which Dr. Walker ob-

[1] Studies on virus diseases of fish. I. Lymphocystis disease of the Orange filefish (*Aleutera schoepfii*) appeared in the *Amer. Jour. Hygiene,* Vol. 28, No. 3, 455–462, November, 1938.

[2] Member of The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

[3] Abstract in *Anat. Rec.,* **70**: No. 1, Suppl. No. 1, 68.

served such a tumor. The tumor, preserved with Bouin's fluid and stained with Harris' hematoxylin, consists of large cells of diameters of 135 to 225 microns, located in the connective tissue of the fin membrane.

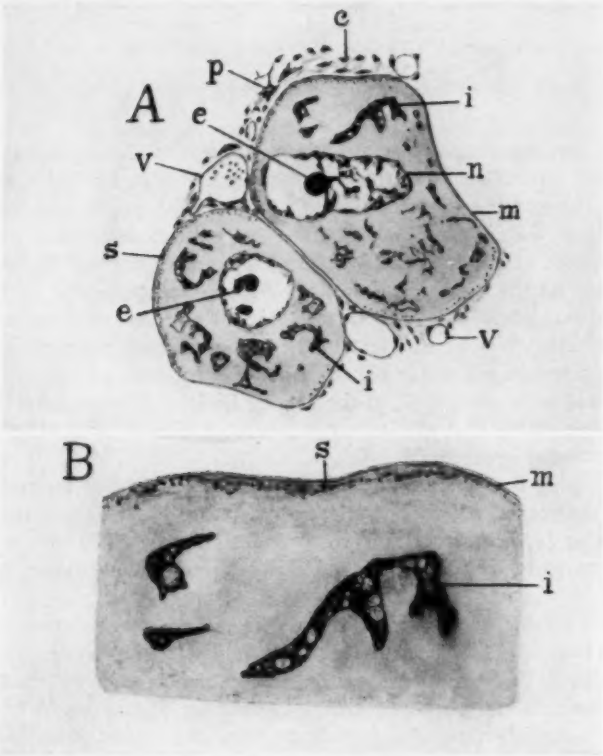

FIG. 1. *A*. Two lymphocystis cells of *Fundulus heteroclitus*. × 210. *B*. Upper part of the upper cell with higher magnification. × 600. *n*, nucleus; *e*, nucleolus; *i*, inclusion bodies; *m*, membrane; *s*, strongly stained granules in the cortex of the lymphocystis cells; *c*, small connective tissue cells; *p*, pigment cell; *v*, vessels.

These large cells (Fig. 1*A*) contain in their cytoplasm the peculiar reticular inclusion bodies which represent the most conspicuous structural element of lymphocystis cells. The inclusions (*i*) appear in the sections as separate coils, but are probably part of a continuous network, similar to those found in the lymphocystis cells of the perches

(*Acerina, Stizostedion*). Figure 1*B* shows with higher magnification their basophilic framework representing a reticular or alveolar structure, strongly stained with hematoxylin. The meshes or alveoles are filled with a paler tingible substance.

These inclusion bodies are so characteristic that the diagnosis of lymphocystis cells is assured without further ado although the second typical structure of the lymphocystis cells, the cell-membrane, shows a remarkably poor development. The cell-membrane appears in Fig. 1*A*

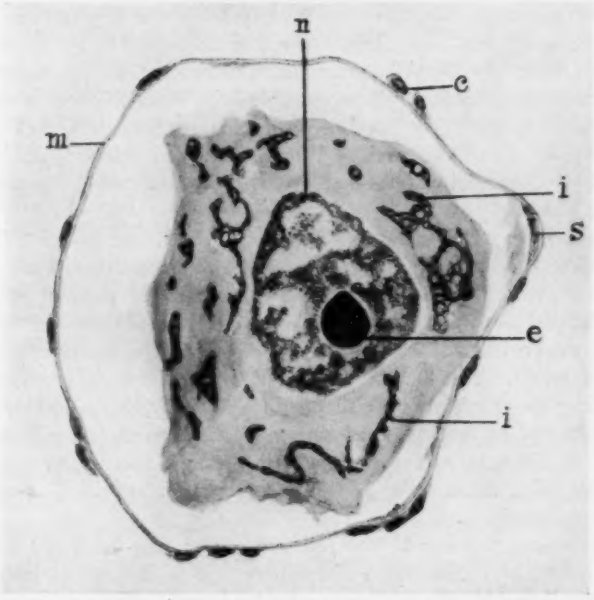

FIG. 2. Lymphocystis cell of *Fundulus heteroclitus*, the cytoplasm of which has withdrawn by shrinking from the membrane (*m*). *n*, nucleus; *e*, nucleolus; *i*, inclusion bodies; *s*, strongly stained granules in the cortex of the lymphocystis cell; *c*, small connective tissue cells. × 600.

only as a contour (*m*) and also with higher magnification (Fig. 1*B*), the configuration of the membrane is not very clear. But cells, in which the cytoplasm has withdrawn from the membrane by shrinking (Fig. 2), show distinctly that the membrane is represented by a cuticula of double contour, evidently homologous to the thicker membranes of the lymphocystis cells of other fish.

The nuclei contain in their peripheral zone a fairly large amount of chromatic substance as bars and fields of granules. One or two nucleoli

(*e*) are to be seen which, as it seems, can give origin to buds (Fig. 1*A*). Just beneath the membrane of the lymphocystis cells a layer of irregular granules (*s*), strongly stained with hematoxylin, could be observed, which, as a rule (Fig. 1*A*), have only developed on those planes of the cells which face the surfaces of the fin membrane. Something similar was seen by the author hitherto only on some degenerating lymphocystis cells of *Pleuronectes flesus*, but there in the whole peripheral zone of the cells.

Because it would be very important for the further research on lymphocystis virus to obtain a stock of lymphocystis-affected fish which might be easily kept in aquaria, the writer attempted to pursue in 1938 the trail found by Dr. Walker. Unfortunately, it seems that the occurrence of lymphocystis disease on *Fundulus heteroclitus* is very rare. Five hundred and fifty specimens of this species, preserved at the Marine Biological Laboratory, Woods Hole, in August 1938, were thoroughly examined without discovering any other case of lymphocystis disease.

Further, there was no success in attempting to transmit to *Fundulus heteroclitus* the lymphocystis disease of the wall-eyed pike perch, *Stizostedion vitreum*, fresh material of which could be procured from the Great Lakes. Corresponding experiments to transmit the *Stizostedion*-disease to the freshwater killifish, *Fundulus diaphanus*, gave the same negative result. The method applied in these experiments consisted in dispersing an emulsion of *Stizostedion*-lymphocystis tumors into the aquarium and in feeding small pieces of the tumors. The *Fundulus*-fish of both species were very eager to swallow the *Stizostedion*-lymphocystis cells, but none of 16 *F. heteroclitus* and of 12 *F. diaphanus* became infected.

Concerning this result, the following facts may be taken into account: (1) The transmission of lymphocystis disease under laboratory conditions was hitherto only successful by keeping diseased and healthy fish together or by the method applied in the *Stizostedion-Fundulus*-experiments which attempted to imitate the natural conditions of infection. (2) This method gave, as a rule, positive results in high percentage in all experiments in which the applied tumor material originated in the same kind of fish to which the treated fish belonged. [See experiments of Weissenberg in Europe on *Acerina cernua* (1914)[4] and *Pleuronectes flesus* (1921)[5] and in this country on *Stizostedion*

[4] Weissenberg, R., 1914. Über infectiöse Zellhypertrophie bei Fischen (Lymphocystiserkrankung). *Sitzungsber. d. Kgl. preuss. Akad. d. Wiss., Physik. mathem. Kl.*, **30**: 792–804.

[5] Weissenberg, R., 1921. Lymphocystiskrankheit der Fische. *Handbuch der Pathogenen Protozoen herausgegeben von v. Prowazek-Nöller*, **3**: 1344–1380.

vitreum (still unpublished)]. (3) The lymphocystis disease of *Acerina cernua* was discovered by Weissenberg on a Baltic Sea tribe of this species. A freshwater tribe of the same species showed in an infection experiment a lower susceptibility than the original tribe (Weissenberg, 1914, l.c., p. 802). (4) Experiments to infect fishes of other species, genera or families by keeping them together with lymphocystis-affected fish has hitherto always given negative results. But these observations (Joseph, 1918;[6] Weissenberg, 1921, l.c., p. 1348) referred to fish on which lymphocystis disease is not observed in nature.

The negative result in the aforesaid experiments to transmit lymphocystis disease from *Stizostedion vitreum* to *Fundulus heteroclitus* and *diaphanus,* in which experiments a certain susceptibility for the disease could be supposed at least for *F. heteroclitus* on account of the described case of Woods Hole, is in accordance with the interpretation that different kinds of lymphocystis viruses are to be distinguished which are adapted to different fishes.

It would be very desirable to pay attention to further cases of lymphocystis disease of *Fundulus heteroclitus* and to keep affected specimens together with healthy fish of this species for cultivating a stock of lymphocystis-affected fish, which could easily be kept under laboratory conditions and therefore would be well adapted for the further study of this interesting virus disease in this country.

Summary

The first and until now solitary case of lymphocystis disease in *Fundulus heteroclitus* is described. *Fundulus heteroclitus* and *F. diaphanus* proved not susceptible to the virus of the lymphocystis disease of *Stizostedion vitreum* in infection experiments.

[6] Joseph, H., 1918. Untersuchungen über Lymphocystis Woode. *Arch. f. Protist.*, Bd. **38.**

DIURNAL VERTICAL MIGRATIONS OF DEEP-WATER PLANKTON

TALBOT H. WATERMAN, RUDOLF F. NUNNEMACHER, FENNER A. CHACE, JR., AND GEORGE L. CLARKE

(*From the Woods Hole Oceanographic Institution*[1] *and the Biological Laboratories, Harvard University*)

Introduction

Diurnal vertical migrations have long been known to play an important part in the lives of pelagic organisms. The numerous previous studies on these migrations in oceanic animals have been limited almost entirely to plankton living relatively near the surface. It was the purpose of the present investigation to continue and extend the observations made by Welsh, Chace, and Nunnemacher (1937) on the diurnal vertical migrations of deep-water animals. More specifically, our objectives were (1) to ascertain the vertical extent of these migrations for the bathyplankton,[2] (2) to determine the greatest depth in the sea at which these migrations still occur, and (3) to discover the relations of these phenomena to the changes in submarine illumination with time and depth.

Historical Considerations

The earliest knowledge of diurnal vertical migrations of marine organisms arose from the practical observations of fishermen. Thus man has known from time immemorial that under certain conditions if he wishes to obtain a good catch of herring, his nets should be set deep in the water during the daytime and shallower at night. Such observations were abundantly confirmed and extended for shallow-water animals by the work of a great many marine biologists (an extensive literature is cited by Rose, 1925; Russell, 1927; and Clarke, 1933*b*).

As far as the plankton of deeper water was concerned, a small amount of information was also already at hand by quite an early date. For example Rang (1828) observed that the pteropod, *Hyalocylis striata*, among others, could be taken at the surface of the sea only after sunset and before sunrise and sank into deeper water during the day. Recently Stubbings (1938) has found that this species descends during

[1] Contribution No. 205, Woods Hole Oceanographic Institution.

[2] See Ekman ('35, pp. 451–77) for a comprehensive general discussion of the bathypelagic fauna.

the day to 200–250 m. so that for at least part of the day it is a bathypelagic organism, i.e., one which lives in the intermediate depths rather than near the surface or the bottom of the sea. But the significance of this and other similar early observations was not appreciated until long afterwards since the existence of a bathyplankton had not been realized until the latter part of the nineteenth century when the data of the various great oceanographic expeditions were analyzed.

Murray (1885, p. 218) concluded from his experiences on the "Challenger" that, "the great majority of plankton organisms live at various depths down to and even deeper than 100 fathoms during the day . . . and only come to the surface at night. . . ." Other investigators came to similar general conclusions (e.g., Chun, 1890, pp. 82–4; Brauer, 1906, p. 337; 1908, p. 232). Brauer was among the first to point out the possible relation of these migrations to such interesting biological problems as bioluminescence, type and size of eye, and pigmentation in the bathyplankton.[3] However, it remained for Fowler (1905, 1909) first to demonstrate by means of numerical data for schizopod and ostracod crustaceans the diurnal vertical migrations of moderately deep-living species. Soon afterwards Murray and Hjort (1912) showed clearly that such migrations were undertaken by organisms which were definitely "deep-sea" animals during the daytime; these investigators also emphasized the correlation of these phenomena with the general biology of the sea, sustaining and extending some of Brauer's suggestions by means of their more complete data.

The further inquiry into the exact and quantitative aspects of the subject presented considerable technical difficulties since for this purpose it is necessary to know accurately where the plankton has been caught, which is impossible when working with open nets. Hence the precise determination of the vertical extent of diurnal migrations and of the various depths at which they occur had to await the development of efficient deep-sea equipment for sampling populations at known depths without contamination from other strata.

Since the invention of the Sigsbee gravitating deep-sea trap (1880) and Palumbo's closing net (Chierchia, 1885, p. 81), many oceanographers have devised such apparatus; yet it was only very recently that reliable equipment has been available for these purposes.

Methods

The observations were carried out in July, 1937, at "Atlantis" Station 2894 (39° 06′ N., 70° 16′ W.) which is in continental slope

[3] For recent investigations of these problems see Welsh and Chace, 1937, 1938.

water about 300 miles east of Cape May, N. J. This position was chosen for several reasons: (1) The slope water, except for the superficial layers, is relatively free from currents (see Iselin, 1936, p. 11). (2) The station was far enough from both the Gulf Stream and coastal waters to be free of their influence. (3) The water was sufficiently deep to prevent benthonic organisms from confusing the results. (4) Data from the continental side of the Gulf Stream might offer some interesting points for comparison with results previously obtained (Welsh, Chace, and Nunnemacher, 1937) in the far more transparent waters of the Sargasso Sea.

In order to keep the ship in about the same geographic position the hauls were made in various directions while the work was being done. The maximum deviation from the original station was about 14 miles (see Table I).

TABLE I

Position of the ship at various times while the work was being carried out. The first position in the table is that designated as Station 2894.

Date	Time	Latitude	Longitude
July 18th	10 P.M.	39°06′ N.	70°16′ W.
19th	5 A.M.	38°57′	70°30′
19th	Noon	39°01′	70°30′
20th	Noon	39°06′	70°33′
21st	Noon	39°00′	70°30′
22nd	Noon	38°59′	70°30′

Horizontally hauled closing nets were used, modified from those employed by Leavitt (1935, 1938) as described by Welsh, Chace, and Nunnemacher (1937). These were 2 m. in diameter at the mouth and 9 m. long; the sides were of stramin, 6 threads to the centimeter, with a silk inset, about 20 threads to the centimeter (54 to the inch), in the tail. While the nets were open, the speed of the ship relative to the surface water was kept close to 2 knots. However, the actual speed of each net in relation to the water layer through which it was moving was not known. Since the water at various depths was probably moving at different velocities and possibly even in different directions, the results in such a situation might be very misleading as to the actual population distributions. But in the present case the fact that the hauls were made in various directions precludes the origin of systematic errors from this source. Furthermore, the evidence from the catches for a regular diurnal migration in the various organisms indicates that the relative

movements of the water were not of sufficient magnitude to confuse the results.

The depth at which a net was fishing was estimated by means of the trigonometric relation between wire angle and the amount of cable out. At 1,000 m. this estimate was probably accurate within about 10 per cent, being more accurate in shallower and less so in deeper tows.

Thirty-nine catches in a series of 16 hauls were made with the nets fishing for 2 hours at various depths and times throughout more than 2 diurnal cycles. Each entire haul took 4 hours so that 6 were made in a 24-hour period. Tows were made at 100, 200, 400, 600, 800, 1,000 and 1,200 meters, calculated depth, and since the bottom was at 2,860 m., the population thus sampled was purely pelagic. Although the closing nets worked very well in general, 4 of the 39 hauls failed; these are marked in Fig. 1 by asterisks.

Figure 1 represents graphically the times and depths of all the closing net hauls made at this station and shows that various depths were sampled on succeeding days. During the morning of the third day on station a series of tows at 100 and 1,200 m. was begun, but the sea became too rough to continue towing (force 5 on the Beaufort scale) so only one haul was made before work was stopped. In an attempt to fill in the gaps in our series we made tows at appropriate depths at noon and midnight on July 22, i.e., about 36 and 48 hours after the continuous series of hauls had been abandoned.

Throughout the periods when the plankton hauls were being made, a record was kept of the light intensity on deck; furthermore, two series of measurements of submarine illumination were carried out. The same submarine and deck photometer cases were employed and the same procedure followed as previously described by Clarke (1933*a*, 1938*a*).[4] However, the Westinghouse "photox" cells in his apparatus were replaced by emission photoelectric cells of the "photronic" type (manufactured by the Weston Electrical Instrument Corp.) since the former had been found to be somewhat variable. To measure temperature changes, a small thermometer was installed inside the case of the deck photometer, beside the cell, where it could be read through the photometer window by raising the opal glass momentarily. The temperature inside the photometer case during the day was found to vary between a minimum of 19° C. and a maximum of 35° C. Correction factors throughout this temperature range were determined from tests carried out in the laboratory at the end of the cruise, in collaboration with

[4] In making these measurements the shadow of the hull is avoided by heaving the ship to with her stern toward the sun and suspending the sea photometer from the end of the mizzen boom (compare criticism by Poole, 1938).

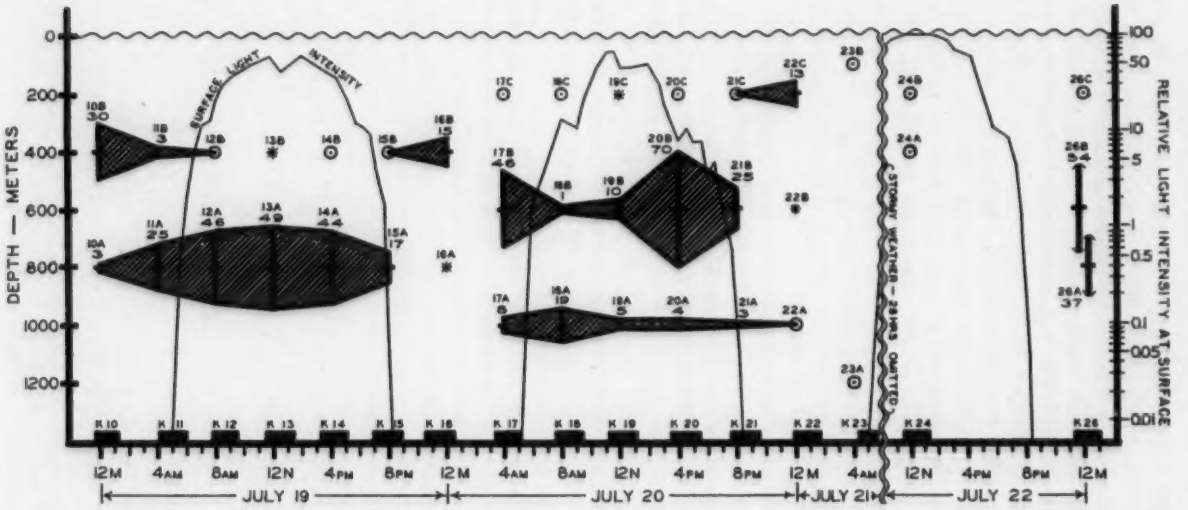

FIG. 1. Distribution in depth and time of the closing net hauls at Station 2894. The numbers of the hauls and their duration are shown by the heavy bars along the time axis. The numbers of the individual nets and their depth are indicated by the small numerals (10*A*, 10*B*, etc.) above the histograms. Those catches which failed are denoted by asterisks. The larger numerals and the vertical bars of the histograms represent the number of adult *Gennadas elegans* taken in each net. Hauls which contained none are designated by an open circle. The changes in the relative surface light intensity are also shown.

Dr. W. R. Sawyer. The proper correction, varying up to a maximum of 12½ per cent, has been applied to each of the photometer readings.

In the first series of measurements of the penetration of light into the water (Series 450) the photronic cell was used with opal diffusing disc, but no filter. Thus the spectral sensitivity of the photometer was that of the photronic cell itself (See Weston Electrical Instrument Corp. Circ. TD–10), the sensitivity of which reached a maximum at 5800 A. and dropped off to 10 per cent of this value at 7080 A. and 3420 A. In the second series (Series 451) the "green" filter combination (Schott-Jena BG–18 and GG–11) used previously and the opal disc were employed. The transmission of this green filter combination exhibited a maximum at about 5100 A. and fell to 10 per cent of this value at about 6460 A. and 4640 A. When these filters were used their selective action was superimposed upon that of the photronic cell with the result that the point of maximum sensitivity of the whole instrument fell between 5800 A. and 5100 A.

Results

This paper presents the results on the occurrence and diurnal vertical migrations of the eleven most numerous malacostracan crustaceans. The decapod, *Gennadas elegans* (Smith), has been chosen for detailed analysis of the data since its presence in significant numbers (526 specimens), its apparent freedom from marked swarming, and its definite diurnal migrations make it a favorable form for our purpose. This prawn was taken at all depths except 100 and 1,200 m. although 83 per cent of the adults captured occurred at 600 and 800 m.

Figure 1 shows that during the first day the number of *Gennadas* present at 800 m. showed a 15-fold increase between midnight and 8 A.M., followed by a further small increase by noon. By 4 P.M. the population present at this depth had decreased to 35 per cent of the number there at noon. Since *Gennadas* disappeared entirely from 400 m. or shallower after 4 A.M. and was not found at this depth again until midnight, the catches strongly suggest that individuals appearing at 800 m. between 4 A.M. and noon had migrated down from lesser depths. The series of hauls at 200, 600, and 1,000 m. on the second day confirmed this indication by showing that the *Gennadas* stock which was present at 200 m. at midnight moved downward a considerable distance during the morning and returned again to shallow water during the afternoon and evening. The numbers taken at 600 m. at various hours of the day suggest further that the bulk of the *Gennadas* stock has gone below 600 m. at the deepest extent of this vertical migration. It is noteworthy that the only hour at which these crus-

taceans were taken at 200 m. was midnight when a significant number of them were taken at that depth. Clearly a shifting of the population of this species by nearly 400 m. took place on both days when hauls were made.

The conclusions drawn from the above horizontal histograms (Fig. 1) appear even more clearly when the results of the different days are combined and regraphed in the conventional vertical histograms (Fig. 2). It is furthermore apparent from this graph that the vertical spread of the population remained about the same throughout the 24 hours in spite of its bodily translation up and down. Hence these migrations involved a concerted vertical movement of the population as a whole rather than massing and scattering effects due to relative movements between various parts of it.

One of the more striking features of the behavior of the *Gennadas* population shown in Fig. 2 is that the animals were apparently not at the same depths at each midnight. Examination of Fig. 2 shows that the *Gennadas* as a whole are considerably deeper in the water in the second midnight histogram than they are in that for the first midnight. Reference to Fig. 1 demonstrates that the difference between these two histograms depends on the deeper distribution of this prawn on the midnight following the storm of the twenty-first than at the same hour on the preceding days. Specifically, on the midnight of the fourth day none were taken at 200 m. in contrast to the 13 taken there two days before, whereas twelve times as many were taken on this occasion at 800 m. as were taken at the same time and depth four days before.

Our data yield some further details concerning the behavior of *Gennadas elegans*. There were no significant differences in vertical distribution and migration between the males and females (adult). On the other hand, there were notable differences appearing between the behavior of the adult and the post-larval specimens. All of the 67 post-larvae of *Gennadas* taken in our hauls were caught at 600 m.; none appeared at any other depths. Thus, if the post-larvae underwent diurnal migrations, the vertical extent of these was not great enough to bring the animals at any time, in sufficient quantities to be caught, within strata sampled by the other nets.

The evidence at hand for *Hymenodora glacialis* (Buchholz) is unfortunately more circumstantial than conclusive (Fig. 3*A*). Adults were taken only at 1,000 m. and in small numbers; from these facts and from the results of previous work (see Welsh and Chace, 1937, p. 63) it is clear that the adults of *Hymenodora* live generally deeper in the water than our nets fished. The post-larval and immature specimens of this crustacean were living shallower so that a considerable

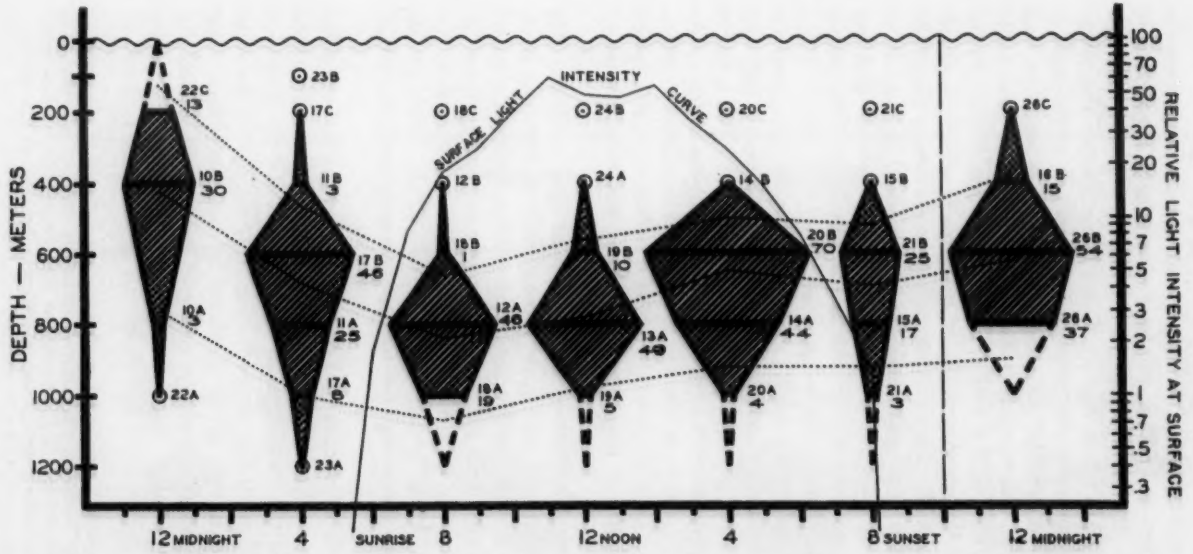

FIG. 2. *Gennadas elegans:* distribution of the adults in the various catches so arranged as to represent the position of the population at the different hours of the day. The small numerals (*22C*, *10B*, etc.) to the right of the histograms are the numbers of the nets, which are given so that the method of combining the data of the different days will be clear from a comparison with Fig. 1. The vertical dashed line separating the second midnight histogram from the rest emphasizes the fact that 3 of the 4 nets in the group were hauled after the storm of July 21. The horizontal bars of the histograms and the larger numerals represent the numbers of animals. The upper and lower dotted lines delimit boundaries of 80 per cent of the population and the middle dotted line represents its center. The light intensity curve is an average for the 4 days measured.

number of them was captured. Sixty per cent of the 153 specimens in the hauls were taken at 1,000 m. and none were caught at any time of day above 800 m. It is unfortunate that the 600 m. net on the midnight of July 20–21 failed to fish so that we do not know whether this prawn was present at this crucial point or not. It we assume on the basis of evidence afforded by some of the other crustaceans, as we have done above for *Gennadas,* that the population was about 200 m. lower in the water on July 22 than on the days preceding the storm of July 21, we can conclude that *Hymenodora* probably was present at 600 m. on the midnight of July 20–21. Owing to the lack of hauls deeper than 1,000 m. during the middle of the day, the lower boundaries of five out of the seven histograms are indeterminate. Indecisive as the present data may be, it is probable, nevertheless, that this animal was undertaking diurnal vertical migrations of about 200 m. amplitude.

Parapasiphaë sulcatifrons Smith (Fig. 3*B*) was the next most numerous decapod crustacean in our catches after *Gennadas* and *Hymenodora.* Fifty-nine per cent of the 123 specimens [5] taken occurred at 800 m. and 10 per cent of them at 1,000 m. Furthermore, this prawn was never caught at 200 m. at any time of day and was taken at 400 m. only at midnight. Thus the general vertical distribution of this species was appreciably deeper in the water than that of *Gennadas* but not of *Hymenodora.*

A diurnal vertical migration of *Parapasiphaë* also occurred. The downward movement was essentially accomplished in the four hours from midnight to 4 A.M. and the upward migration apparently started slowly in the late afternoon although 75 per cent of its extent was accomplished more rapidly after 8 P.M. The fact that these animals, too, were considerably lower in the water on July 22 is shown by the differences between the histograms for the first and second midnights in Fig. 3*B*.

Only one of the six species of *Sergestes, S. arcticus* Kröyer, taken at this station occurred in sufficient numbers for our present purpose. An examination of the distribution in the catches of the 104 specimens of carapace length of 10 mm. or over (Fig. 3*C*) shows that the older stages of this prawn were living shallower in the water than either *Parapasiphaë* or *Gennadas* since the center of the average vertical distribution of the population was just above 600 m. rather than about 800 m. as it was for the other two forms. These *Sergestes,* moreover,

[5] Immature, post-larval, and larval specimens were included in this count as well as in the figure. Although the immature specimens were not identified as to species, they were included here as *P. sulcatifrons* since all individuals whose development was sufficiently advanced to permit identification belonged to this species.

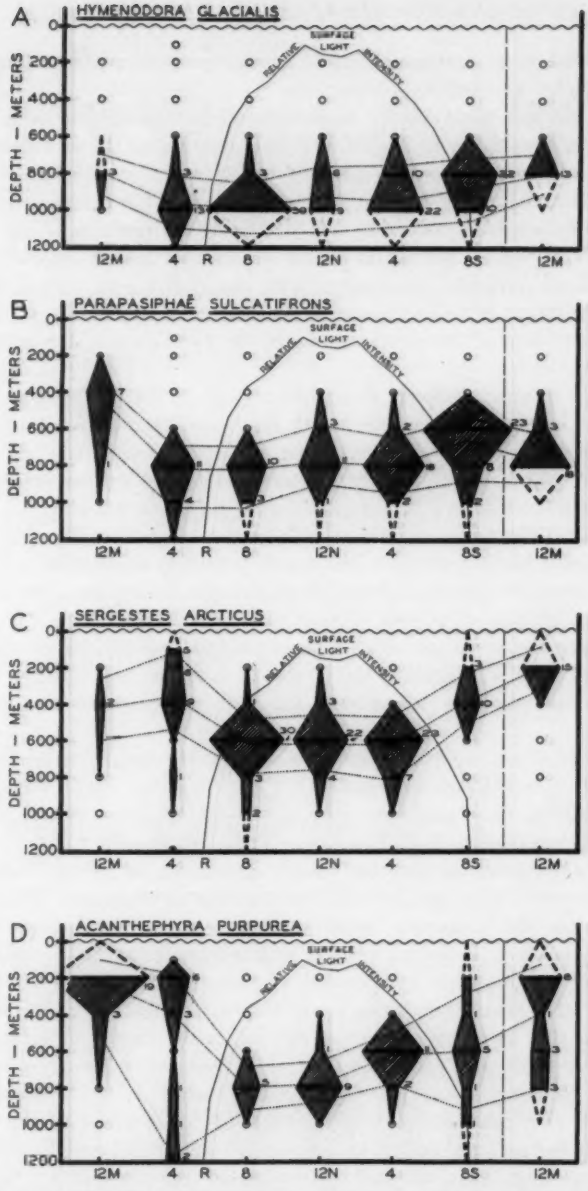

FIG. 3. Decapods: data condensed as in Fig. 2. The numerals to the right of the histograms represent the numbers of specimens taken. These figures include: *A*, larval, post-larval, and immature stages; *B*, all stages; *C* and *D*, adults.

also exhibited an extensive diurnal vertical migration of at least 200 and probably as much as 400 m.

The absence of significant numbers of the species in the hauls made for the first midnight of Fig. 3*C* was probably due to the fact that most of the population was in less than 200 m. of water at this time. Some circumstantial support is given this explanation by the presence of some of these decapods at 100 m. at 4 A.M. and by the position of the population shown by the histogram of the second midnight in Fig. 3*C* since the stock of *Sergestes* by analogy with observations on several of the other Crustacea would be lower in the water by about 200 m. than it would have been at the same time under the conditions of the preceding days.

There is some evidence from an analysis of the catches that those *Sergestes arcticus* of carapace length less than 10 mm. live at and migrate to slightly shallower levels than the larger individuals shown in the figure and discussed above, but hauls closer together than ours would have been necessary to determine this relation quantitatively.

Only 68 *Acanthephyra purpurea* A. Milne-Edwards (Fig. 3*D*) were taken in our hauls, and these had a rather patchy distribution. Nevertheless, there is some definite evidence for the occurrence of a diurnal vertical migration of at least 400 and possibly 600 m.

Among the more primitive malacostracans two species of euphausiids, two species of mysids, and two species of amphipods were caught in sufficient numbers to provide adequate samples for the present analysis.

The relatively enormous number of 180,000 specimens of the euphausiid, *Nematoscelis megalops* G. O. Sars (Fig. 4*A*), was taken. The 1,543 adults among these were living mainly between 400 m. and the surface although 12 of them were taken in the only 1,200 m. haul. From the evidence of the very patchy occurrence of all the *Nematoscelis*, one would conclude that this species swarms markedly, which may account for the extremely small numbers caught in the noon hauls. Clearly, however, a diurnal vertical migration at least 200 m. in extent was being accomplished by the adult *Nematoscelis*, shown in the figure.

The other schizopods taken in significant numbers were also migrating vertically, as much perhaps as 600 m. in the case of *Thysanopoda acutifrons* Holt and Tattersall (Fig. 4*B*), and 400 m. in those of the mysids, *Boreomysis microps* G. O. Sars (Fig. 4*C*), and *Eucopia unguiculata* (Willemoës-Suhm) (Fig. 4*D*). A comparison of the histograms of the first and second midnights in Figs. 4*B*, *C*, and *D*, indicates that these organisms, as well as several of the previously discussed Crustacea, were 200 m. deeper in the water after the storm of July 21

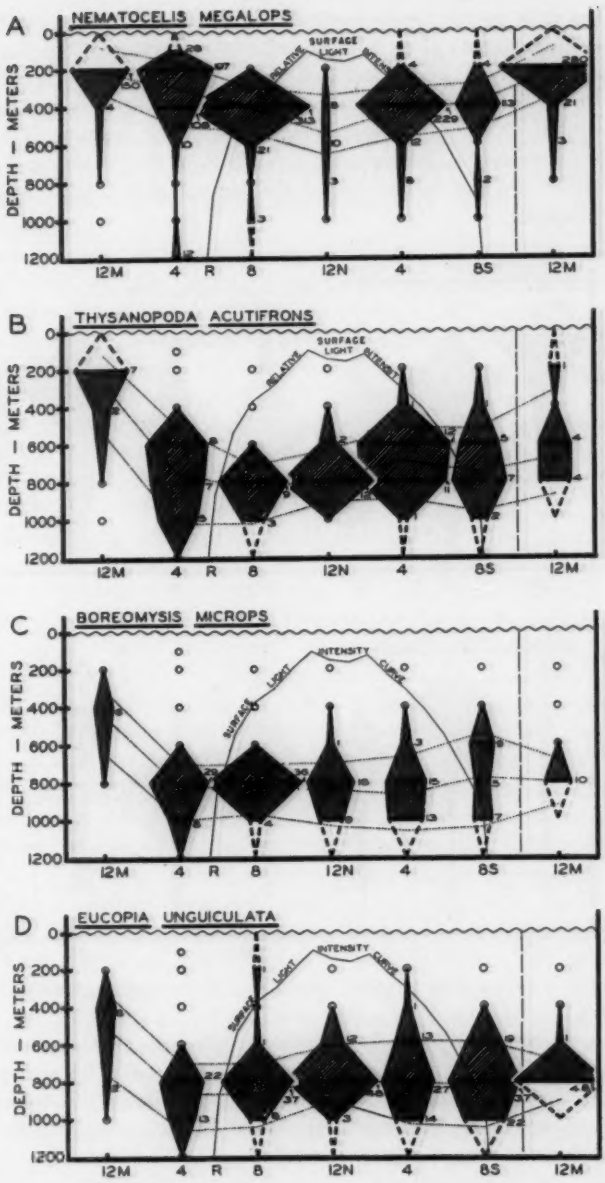

FIG. 4. Euphausiids and mysids. *A*, large adults; *B* and *C*, adults; *D*, all stages. See Fig. 2 for further explanation.

than they were on the previous two days. It is furthermore noteworthy that the *Thysanopoda* (Fig. 4*B*), as well as the *Gennadas* (Fig. 2), *Parapasiphaë* (Fig. 3*A*), and *Acanthephyra* (Fig. 3*D*) apparently began to move slowly upwards in the water almost as soon as they had reached the lowest point of its downward excursion in the morning; this slow upward migration was succeeded at about 8 P.M. by a more rapid movement which brought the animal to the highest point of its migration about midnight.

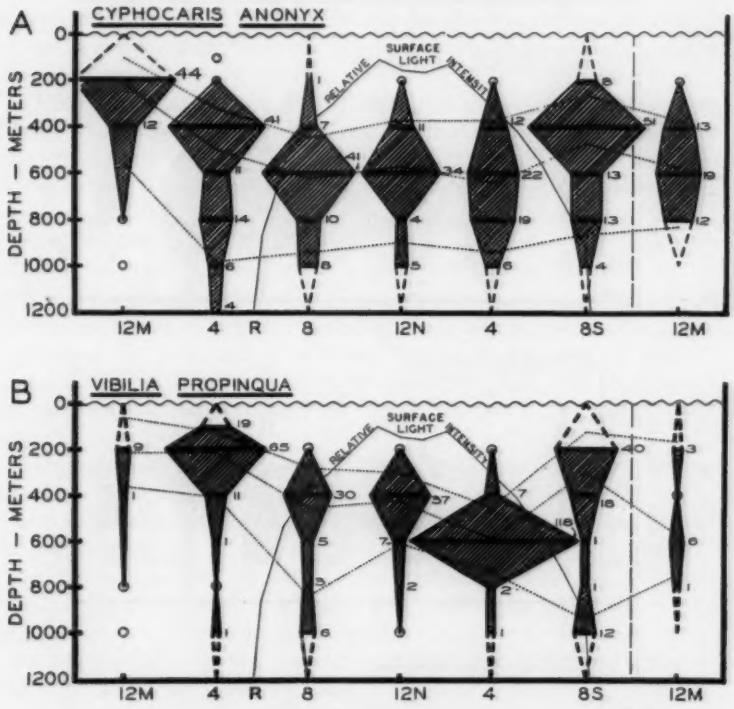

FIG. 5. Amphipods. Adults. See Fig. 2 for further explanation.

The two amphipods caught in sufficient numbers, *Cyphocaris anonyx* Boeck (Fig. 5*A*) and *Vibilia propinqua* Stebbing (Fig. 5*B*), also show a diurnal vertical migration of about 400 m. amplitude. In the case of *Vibilia,* however, the considerable amount of swarming which is apparent obscures this migration somewhat.

Discussion

Since the hauls were made in such a way that our data give us information about points separated in effect by 200 m. and 4 hours, a detailed analysis of the diurnal vertical migrations is clearly impossible. Nevertheless, our results show that all of the crustaceans investigated were accomplishing vertical migrations 200 to 400, and possibly 600 m. in extent, which is in general agreement with the results of recent investigators.

For instance, certain of the larval stages of euphausiids taken by Fraser (1936, p. 154) were apparently moving upward 200–250 m. to reach the surface at night. Stubbings (1938, p. 27) found that species of the pteropods, *Creseis* and *Hyalocylis,* underwent a diurnal migration of 200 m. vertical extent and that some of the other pteropods may have been migrating even further. The copepod, *Pleuromamma robusta,* was shown by Mackintosh (1934, p. 92) to exhibit the most pronounced vertical migration of all the plankton animals of the antarctic surface waters; this crustacean rose into the upper 100 m. around midnight and descended apparently below 600 m. during the day. Among the malacostracan crustaceans Hardy and Gunther (1935, p. 240) observed that *Euphausia frigida* and *E. triacantha* underwent a diurnal vertical migration of at least 200 m. and that the amphipod, *Parathemisto gaudichaudi,* migrated a vertical distance of about 60 m. in the course of its diurnal movement. Since the latter animal was taken within the upper 100 m. at all hours, this observation is similar to those made on other shallow water plankton. Clarke (1934a, Figs. 2 and 3), for example, showed that *Calanus finmarchicus* adults and copepodid stages IV and V exhibited a diurnal migration of about 100 m.

The rate of the diurnal vertical migrations may be roughly calculated from our results. If the speeds of downward movement of the centers of the populations for the various species, as shown in the figures, are taken to represent the average rates of movement for the organisms concerned, these are found to vary from 29 m. per hour for *Nematoscelis* to 125 m. per hour for *Thysanopoda.* The average for the eleven species of crustaceans is 67 m. per hour. The only comparable data are those of Hardy and Gunther (1935, p. 240) mentioned above for the migration of the two species of *Euphausia* in which the upward migration apparently occurred at a rate of 100–200 m. per hour.

In some of these organisms a slower upward movement during the day preceded the more rapid upward migration shortly before midnight. This phenomenon has been observed in shallow-water zoöplankton by a number of workers and has been explained on the basis of a gradual

light adaptation of the animals (see Russell, 1931, p. 402; Kikuchi, 1938, p. 37). In spite of this slow upward trend, however, our observations suggest that the upward migration in the early evening hours was as rapid as the downward movement in the early morning.

A comparison of the swimming speeds of the animals in the plankton with the rates of these diurnal vertical migrations would be of great interest. Unfortunately, there are no data available for these or comparable plankton organisms. Measurements have been made, however, of the rates of locomotion of several copepods and crustacean larvae (Russell, 1927, p. 221; Welsh, 1933). The latter found that *Centropages* would swim towards a light of an intensity that might be found 20–30 m. below the surface on a clear day at a rate of 82 m. per hour (actually measured over a path only 10 cm. long). As Parker (1902), Gardiner (1933), and Seiwell and Seiwell (1938) have shown, gravity alone causes copepods to sink at a rate of about 24 m. per hour.

Thus, if any organism to which the above quoted rate of sinking is applicable were migrating downward 400 m. in 8 hours, it would sink 192 m. merely through the effect of gravity and would have to swim downward through the remaining 208 m. at an average rate of 26 m. per hour. For the upward trip the gravitational effect would add the 192 m. to the distance to be swum (see Clarke, 1934*b*) so that the animal would have, in effect, to swim upward at a rate of 592 m. in 8 hours or 74 m. per hour. To consider it in another way, the organism in order to accomplish the downward migration would have to be actively swimming towards the bottom at the experimentally determined rate of 82 m. per hour for only about one-third of the 8-hour period, whereas in order to move upward to the original level in the same length of time it would have to be swimming towards the surface at this rate during 7 of the 8 hours.

As the pelagic malacostracans such as those discussed in this paper are known to be very active swimmers, they could presumably accomplish such vertical migrations with greater ease than the animal postulated above on the basis of data obtained from copepods. The Crustacea in our analysis are larger, moreover, and, other things being equal, would sink more rapidly through the water than the copepods so that an even greater proportion of their downward movement may be effected by the mere absence of any persistently oriented swimming activity. Many experiments have shown that several environmental variables, such as light and temperature, will affect the locomotor activity of zoöplankton, but, as Clarke (1934*b*) pointed out, these factors under natural conditions would seem to work in the wrong direction to explain distribution as it is found.

Since the great majority of previous workers have recognized that light is the most significant single environmental factor in diurnal vertical migrations, it is interesting to correlate these phenomena with the changes in the intensity of illumination at various hours of the day (Fig. 1). During the first hour after daylight became measurable, its intensity increased at an exceedingly rapid rate. Similarly, the illumination diminished at an equally rapid rate during the hour preceding the last measurement of the day. During the four hours in the middle of the day the illumination remained relatively constant and such changes in intensity as resulted from different degrees of cloudiness were of a much lower magnitude than those of early morning and late afternoon.

The most striking point which appears in a comparison of the biological and the light intensity data is that much of the migration apparently occurred when there was no light more intense than starlight even at the surface. Before sunrise most of the animals had already descended about half of their total downward distance, and several, such as *Boreomysis, Eucopia,* and *Parapasiphaë,* had reached the deepest point in their migration by sunrise. In most cases, furthermore, the greater part of the upward migration must have been accomplished between the time of sunset and midnight.

It is clear from these facts that the vertical migrations of these organisms cannot be explained simply on the basis of a negative phototropism which overshadowed a negative geotropism in the daytime and left it with a free hand at night.

Similarly the hypothesis that the animals aggregate by means of a tropistic mechanism (Michael, 1911; Russell, 1934) in a region of optimum light intensity and follow the latter as it moves vertically at various hours of the day apparently will not explain the situation here. Such an hypothesis is further weakened by the lack of evidence in the present case for a general vertical scattering of the population throughout the water column at night when it would be free of the concentrating effect of light.

Whether we assume that external environmental or internal physiological factors are controlling the diurnal migrations, the intensity of illumination penetrating from the surface must play an important rôle in these phenomena since it is the only daily environmental variable in deep water. It may act in combination with other external factors, such as temperature and gravity, since many investigators have shown experimentally that interactions of such factors may cause a tropistic reversal (Kikuchi, 1938, pp. 35–6). Or it may merely keep in phase cyclic internal physiological conditions of the organisms which themselves regulate these migrations (see Russell, 1927, p. 225; Welsh,

Chace, and Nunnemacher, 1937, p. 195). Many cases are known (Welsh, 1938, p. 136) of the persistence of internal diurnal rhythms under constant external conditions, but one would expect that in nature at least an occasional action of the external factor to which it owes its origin, in this case light, would be necessary to maintain the synchrony indefinitely.

A second problem is posed by the fact that most of the Crustacea here considered were about 200 m. lower in the water on the midnight of July 22 than they were at the same hour two or three days before. Furthermore, the maximum illumination at the surface on July 22 was 66 per cent greater than the maximum on July 19. Previous studies have shown that certain organisms appear to live at depths where the light intensity is of a particular magnitude, i.e., an optimum intensity for the organism in question; these organisms may, as a consequence, appear in shallower waters on dull days or in high latitudes (Russell, 1933, p. 569; Murray and Hjort, 1912, p. 664–6); thus the light intensity itself may have been responsible for the observed difference in distribution.

It is clear, however, that the amount of illumination could not explain so great a change in distribution. Since actual measurements down to 84 m. (Fig. 6) showed that the light is reduced by a factor of 10, for every 25 m. of water, the observed change in light intensity at the surface would result in a difference of only about 5.5 m. in the depth at which any given intensity of light would be found. Even in the clearest sea water yet measured (see below) this change in light intensity at the surface would result in a maximum change of only 50 m. in the depth to which any particular intensity of light would penetrate.

An alternative causal or contributory factor to the observed lower position of the plankton on July 22 may have been the storm of the previous day, but it is not likely that a relatively mild disturbance of this sort at the surface would be appreciable at a depth of over 200 m. Furthermore, Hardy and Gunther (1935, p. 268) conclude from their observations that, "... the state of the sea has little or no bearing on the number of organisms in the surface layers. . . ."

A more important point is the fact that simultaneous measurements on the horizontal patchiness of the plankton were not made. Consequently we cannot tell whether or not the observed difference might rather be due to the fact that on the later day we were sampling a different population with a different vertical distribution pattern from that of the previous days (Russell, 1933, p. 569; Hardy, 1936).

One of the most important questions raised by previous work on the diurnal vertical migrations of bathypelagic organisms is how deep

down in the water do such migrations occur. Most of the crustaceans showing diurnal vertical migrations discussed in this paper were living at depths of 600–800 m. during the four hours during the middle of the day. If, as pointed out above, we recognize the necessity of light in one way or another for the vertical migrations of these animals, we must assume that at some time during the day the organism is affected

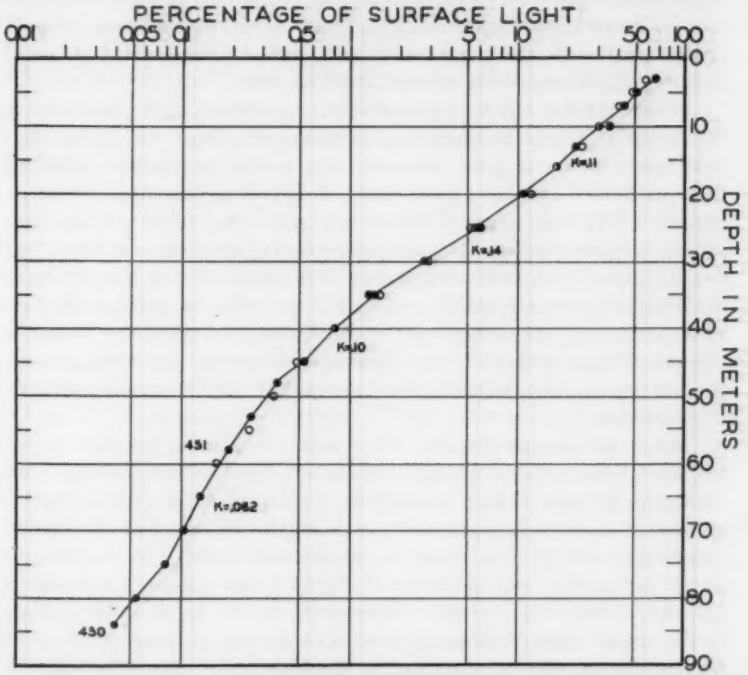

FIG. 6. Relation between depth and irradiation expressed as a percentage of the light just over the surface (logarithmic scale). Station 2894. Lat., 39° 06′ N.; Long., 70° 16′ W. July 22, 1937. Sky: clear. Sea: moderate, white caps. Series 450 measured by photronic cell with opal disc. Series 451 measured by photronic cell with "green" filter combination (Schott-Jena BG–18 and GG–11) and opal disc.

by light. Clearly, under any given physiological conditions, there is a minimum intensity of illumination which would be just sufficient to affect any particular animal; in the open sea there would be a definite maximum depth beyond which this minimum light intensity would never penetrate even at noon on the brightest days. A diurnal vertical migration in which illumination from the surface was supposed to have any

controlling effect would thus be impossible below this depth. As pointed out above, however, vertical migrations could be temporarily maintained under constant conditions by a diurnal physiological rhythm within the organism. Thus, if unusual conditions such as a storm should cut down the incident light so that deep-living animals did not come under the influence of light for several days, there would not be a wholesale loss of the population through continued downward migration or random scattering into unusually great depths (see Russell, 1930), but the diurnal migrations would continue more or less as usual under the guidance of the physiological rhythm.

In view of these facts the question of how much light penetrates to the depths at which the migrating Crustacea are living during the daylight hours is one of great interest. The results of our two series of light penetration measurements, shown in Fig. 6, indicate that the transparency of the water was relatively uniform down to 84 m., the limit of measurement, with an average value of the extinction coefficient [6] of $k = 0.092$ for the green component of daylight. Hence, the intensity of illumination was reduced to one-tenth its value by passage through 25 m. of water. Assuming that the transparency of the water remained the same below the depth of our deepest measurement and extrapolating we find that the illumination at 800 m. would be 10^{-32} times its intensity at the surface.

But if we assume that the water below 100 m. is as clear as any sea water which has been measured, we may on this basis calculate the maximum amount of light possible at any depth. Since the extinction coefficient varies enormously with wave-length, the actual result reached will depend on the part of the spectrum considered. In the clearest water yet known the intensity of light in the green wave-lengths (Clarke, 1938*b*) was reduced to one-tenth by 61 m. of water. Thus if the water below 100 m. at our station was as clear as this, at 800 m. the intensity of illumination would be 10^{-15} times the value at the surface. In the clearest water yet known the blue wave-lengths were reduced to one-tenth their intensity in 230 m. (Clarke, 1933*a*).[7] If water of like transparency existed below 100 m. at our station, the illumination of 800 m. would be a little less than 10^{-7} times the surface intensity;[8] this value is between 100 and 1,000 times higher than the threshold intensity for human vision.

[6] In the equation $I/I_0 = e^{-kL}$, where L is the thickness of the water layer in meters through which the intensity is reduced from I_0 to I.

[7] Basing our calculations on the most transparent strata only.

[8] Assuming the same reductions in the upper 100 m. for the blue component as for the green, which is known (Oster and Clarke, 1935) to be approximately true in this region of the Atlantic.

We have some evidence, however, that conditions as favorable for penetration as this latter case for blue light did not exist at our slope water station since on a bright, calm day Beebe (1934, p. 276) observed through the window of the bathysphere that at a depth of 610 m., the water appeared absolutely dark to the human eye. But Beebe was not able to look upward in the water so that he could not tell at what depth the downward component of the light had become indistinguishable from the blackness below.[9] On the other hand, the water of the Sargasso Sea, where Beebe made his observations, is characteristically (Clarke, 1936*a*) nearly twice as transparent in the upper 100 m. for the green region of the spectrum as was the water at our present station.

It would be of great interest to compare the amounts of light present at 800 m., where migrations were definitely going on, with the visual sensitivity of these Crustacea. Unfortunately, no experimental studies have yet been made on vision in any deep-sea animals. Nevertheless, from previous work on crustaceans and other arthropods with compound eyes, it is possible to draw two extremely general conclusions: first, that arthropods are more sensitive to the blue wave-lengths of light than to the green (see Waterman, 1937, p. 464 for table summarizing pertinent data) and second, that for these organisms the minimal light intensity for response is of the order of magnitude of 10^{-6} to 10^{-7} millilamberts (Crozier, 1938, personal communication).[10]

An intensity of illumination of 10^{-6} millilamberts is equivalent to about 10^{-10} times that of full noon sunlight and is the value used by Clarke (1936*b*) as a threshold intensity for determining the depth at which fish can see. A crustacean with a visual threshold of such a magnitude in the green wave-lengths certainly could not be stimulated by this region of the spectrum when the intensity of the latter is reduced to 10^{-15} times that at the surface. Since this would be the illumination present at 800 m., when we assume that the water below 100 m. was as clear as any sea water that has been observed, we may conclude that under these conditions the crustaceans could not be re-

[9] Presumably this directional effect would still be appreciable at great depths since directional differences in intensity were recorded photographically by Helland-Hansen (Murray and Hjort, 1912, p. 252) at 500 m. and since more recent work of Whitney (1938, p. 211) has demonstrated that in optically homogeneous waters the ratio of the intensities of light passing downward to those of the light scattered upward is a constant (see also Pettersson, 1938). Of interest in this connection is the occurrence of upwardly (dorsally) directed "telescopic" and stalked eyes in a number of different families of deep-sea fishes of the "twilight zone" of the seas (Brauer, 1908, p. 233) which would suggest that this directional difference in the light intensities is of biological significance even at considerable depths.

[10] Any value obtained for a sensory threshold will, of course, depend largely on the exact experimental conditions under which it was determined.

sponding to light from the surface. But if the crustacean had an equally low visual threshold for blue light, our calculations have shown that in the clearest water observed there would be almost 1,000 times as much light present at 800 m. as would be necessary for the threshold of vision. Under such maximum conditions even at 1,000 m., where the immature *Hymenodora* in our catches possibly gave some indications of a diurnal vertical migration, more than 100 times the necessary amount of light would be present. The threshold intensity for vision, 10^{-10} of full sunlight intensity, would occur at a depth of nearly 1,500 m. under such conditions.

Thus, the water below the surface layers could be, as it probably was, considerably less transparent than the clearest sea water ever observed and still permit sufficient visible light for affecting the reactions of bathypelagic organisms to penetrate to depths of about 800 m.

The preceding discussion makes it clear that several important aspects of this problem must be investigated more thoroughly before a satisfactory solution will be possible. These are in brief: (1) actual measurements of the transparency of the water at greater depths and of the relative intensities of the light in various directions; (2) more detailed work on the maximum depth at which diurnal vertical migrations occur; (3) measurements of the photosensitivity of deep-sea animals and correlations of the maximum wave-length sensitivity to the wave-length of the most penetrating part of the spectrum; (4) experimental studies on deep-sea animals designed to elucidate the nature of any diurnal physiological rhythms and their relation to the organisms' behavior in the sea.

Summary

1. A study was made with the aid of closing nets of the diurnal vertical migrations of bathypelagic organisms at a station in continental slope water of the western North Atlantic.

2. While the hauls were being made a continuous record of the light intensity at the surface was kept.

3. The penetration of light into the upper 84 m. of water was directly measured photometrically; the average extinction coefficient for green light was $k = 0.092$.

4. All of the malacostracan Crustacea (to which the detailed results presented in this paper are limited) which occurred in sufficient numbers for analysis exhibited diurnal migrations 200 to possibly 600 m. in vertical extent.

5. The speed of vertical movement in these migrations varied from 24 to 125 m. per hour among the various crustaceans.

6. A considerable part of the migrations took place while the light intensity even at the surface was no greater than starlight.

7. Several Crustacea living at 800 m. during the day showed extensive diurnal vertical migrations.

8. It is concluded, however, that whether the migrations are regulated by external environmental or by internal physiological factors, at some time of day the organisms concerned are affected by light penetrating from the surface.

9. Calculations made from the light penetration data indicate that the amount of light probably present during the middle of the day at the depths where the animals were migrating was adequate to support this conclusion (8).

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THE REPRODUCTION OF LIMACINA RETROVERSA (FLEM.)

SIDNEY C. T. HSIAO

(From the Biological Laboratories, Harvard University, and the Woods Hole Oceanographic Institution,[1] Woods Hole, Massachusetts)

Introduction

The structure of the reproductive system of *Limacina retroversa* and the process of spermatogenesis of this species have been described in a previous paper (Hsiao, 1939). It was shown that very small animals are sexually undifferentiated. The gonads of sexually differentiated but small-sized individuals are protandric and many of them are often without female tissue, that is, "pure male," while those of the bigger animals are essentially hermaphroditic, showing different proportions of male and female tissues. Some individuals function as male, others as female and still others both as male and female at the same time. It is the purpose of this study to examine the relationship between size and sexual phases and the time of the occurrence of the latter so as to determine their sequence in the reproductive history of this form and to compare it with other mollusks whose sexual history is known.

Description of Material

The material was collected by the research vessel "Atlantis" during a series of cruises in the Gulf of Maine between June, 1933 and September, 1934. Specimens collected at the following periods were used:

Date	Designated as
December 2–11, 1933	December specimens
January 9–13, 1934	January specimens
March 21–28, 1934	March specimens
April 17–May 13, 1934	April specimens
May 21–June 2, 1934	May specimens
June 25–July 1, 1934	June specimens
September 17–24, 1934	September specimens

The specimens were caught by vertical hauls made with a 1.5-meter Heligoland larva net drawn from a level near the bottom up to the surface and fixed on board the ship in 2 per cent formalin in sea water.

[1] Contribution No. 206 from the Woods Hole Oceanographic Institution.

Limacina retroversa were later sorted out from the other species and specimens were selected from one to three stations from each cruise so as to make a series covering nearly the whole range of size. Each individual was measured with shell-opening facing the observer under a binocular microscope with a calibrated ocular micrometer. The diameter of the largest whorl of the spiral body of the animal, excluding the protruding margin of the shell-opening (*A–B* in Fig. 1), was measured and used to designate the size of the specimens studied.

FIG. 1. Camera lucida drawing of the shell of *Limacina retroversa*. × 20. *A B:* maximum diameter of the shell used for comparison of size.

From a study of the size distribution of *Limacina retroversa* from 1933–34 and the movement of the water masses, Redfield (1939) has shown that at least two different populations entered the Gulf of Maine during the period of observation. The first group, called "population A," invaded the Gulf during December, 1933 and January, 1934, and later in the spring a second group, "population B," entered the basin during April and May from the same general direction. The positions of the hydrographic stations from which specimens used in this study were taken are shown in Fig. 2. In this map the solid circles with the abbreviations of the months beside them show the position and time of collection of the material from the population of *Limacina retroversa,* which has been identified as "population A" by Redfield. The positions of the stations providing the material fall within the region where large numbers of "population A" were found (as shown by Redfield's map), that is, in or near the center of density of this population in the different months. On the same map (Fig. 2) the open circles, with the abbreviations of the months in parentheses under them, indicate the stations in April and May from which specimens were selected which repre-

sent "population B." From these specimens the reproductive history of the species is worked out. The following table (Table I) shows the number of individuals used from each cruise grouped into size classes with a class interval of 0.3 mm.

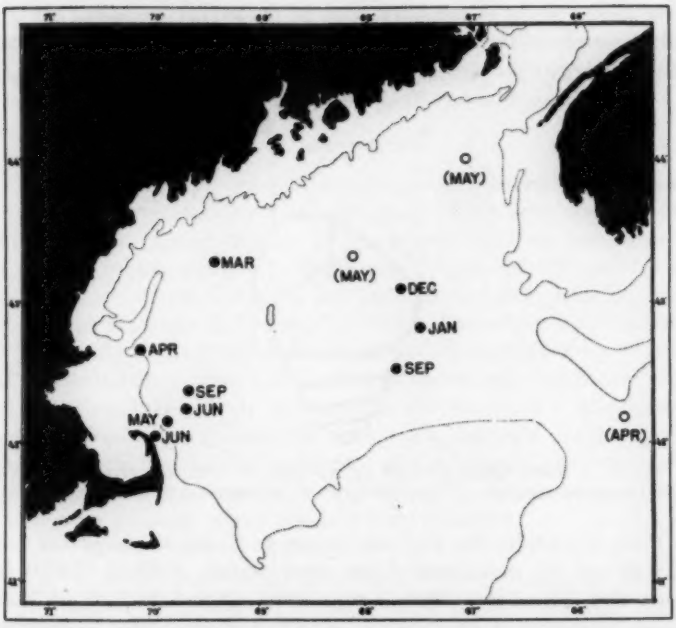

FIG. 2. Map of the Gulf of Maine showing the hydrographic stations from which samples of *Limacina retroversa* are selected for microscopic study.

●: stations for "population A."
○: stations for "population B."

The method employed in making serial sections of these specimens has been described in a previous paper. The following observations are based upon a comparative study of these serial sections.

RELATION BETWEEN SIZE AND SEX

The relation between size and sex in *Limacina retroversa* has been examined from two different angles: (1) The gonadal types and size, and (2) the relation between functional types and size.

Gonadal Types and Size

The following morphological types are seen among *Limacina.* (*a*) Sexually undifferentiated individuals all of which are small-sized; (*b*) "pure males" which are nearly completely male in constitution and are somewhat larger; and (*c*) hermaphrodites, which have various proportions of male and female tissues in the ovotestis and are generally of large size. The occurrence of the different morphological types of individuals in *Limacina retroversa,* in order of size, is shown in Table II.

Sexually Undifferentiated Individuals.—Of all the *Limacina retroversa* sectioned the largest individual which has a gonad still in a sexually undifferentiated condition measures 0.85 mm. in diameter. Individuals larger than this size all show definite gametogenesis. All specimens with a diameter less than 0.6 mm. are sexually undifferentiated. Hence, the process of sexual differentiation must commence during the period when the animal is between 0.6 mm. and 0.85 mm. in diameter. This is brought out more clearly when the percentage of sexually undifferentiated individuals is plotted against size as shown in Fig. 3.

TABLE I

Table showing the number of individuals from each period of collection which were used in this study. The individuals are separated according to size into groups with a class interval of 0.3 mm.

Month of cruise	Size groups in mm.								Total number obtained during month
	0 to 0.3 mm.	0.3 to 0.6 mm.	0.6 to 0.9 mm.	0.9 to 1.2 mm.	1.2 to 1.5 mm.	1.5 to 1.8 mm.	1.8 to 2.1 mm.	2.1 to 2.5 mm.	
Dec.	3	13	9	8					33
Jan.		13	12	4	3				32
Mar.		4	4	7	10	1			26
Apr.			5	9	5	6	1		26
May	4	11	7	6	3	6	2	1	40
June	4	3	6	2	6	5	2		28
Sept.	1	6	8	7	4	3			29

"Pure Males."—These are animals with gonads which contain little or no noticeable amount of ovarian tissue. They correspond to Coe's "true males" or Orton's "pure males." These individuals are confined to comparatively small-sized groups. Table II, column 8, shows the sudden appearance and the steady but more gradual decrease of "pure males" among animals arranged in the order of increasing size. Among larger animals this type of individual gives place to those which have

various proportions of feminine reproductive cells in the gonads. These are the hermaphrodites described in the next paragraph. On the average, about 22 per cent of the specimens less than 1.2 mm. in diameter belong to the "pure male" class. Many of these males are functional at the time of fixation as shown by the presence of mature spermatozoa in the gonoduct.

Animals with Various Proportions of Male and Female Tissues in the Gonads.—In addition to the sexually undifferentiated animals and the "pure males" a small number of animals less than 1 mm. in diameter

TABLE II

Proportion of different morphological types of *Limacina retroversa* separated into groups without regard to the time of collection. Animals smaller than 1.2 mm. in diameter are separated into 0.1 mm. classes, those larger than 1.2 mm., into 0.3 mm. classes.

1	2	3	4	5	6	7	8	9	10
Size in mm.	Total number	Undifferentiated	Pure male	>50% male	<50% male	Percentage undifferentiated	Percentage pure male	Percentage of >50% male	Percentage of <50% male
0.4	3	3	0	0	0	100	0	0	0
0.5	3	3	0	0	0	100	0	0	0
0.6	13	8	3	2	0	62	23	16	0
0.7	15	5	7	3	0	33	47	20	0
0.8	24	3	8	13	0	13	33	54	0
0.9	12	0	3	7	2	0	25	58	17
1.0	14	0	2	8	4	0	14	57	28
1.1	15	0	2	10	3	0	13	67	20
1.2 to 1.49	44	0	0	34	10	0	0	77	23
1.5 to 1.79	34	0	0	26	8	0	0	77	23
1.8 to 2.09	19	0	0	11	8	0	0	58	42
2.1 to 2.39	4	0	0	2	2	0	0	50	50
2.4 to 2.7	2	0	0	1	1	0	0	50	50

may have gonads containing various proportions of male and female tissues at the same time. Of all the animals greater than 1.1 mm. in diameter no undifferentiated or "pure males" were found. They all contain spermary and ovarian tissues in the same gonad. Following the criteria described in a previous paper these hermaphroditic animals can be classified as hermaphroditic males or hermaphroditic females according to whether they have more than 50 per cent or less than 50 per cent of their gonadal tissues consisting of male reproductive elements. The proportion of these two kinds of individuals in the different size groups is

shown in Table II. Column 5 shows the number of individuals with more than 50 per cent of their gonadal tissue made up of masculine elements, excluding those which are already listed in column 4 as "pure males." It shows the number of hermaphroditic males only. The percentages of hermaphroditic males of this class are given in column 9. Column 10 shows the percentages of hermaphroditic females based on the numbers shown in column 6. A comparison of columns 9 and 10 will show that the minimal size of the hermaphroditic females is much greater than that of the hermaphroditic males. This occurrence of hermaphroditic males as well as "pure males" in smaller-sized animals when compared with the hermaphroditic females indicates that the male reproductive tissue is developed when the animals are smaller than is the case with the female cells of reproduction.

Owing to the small number of individuals in each size group among the larger animals the data in the lower half of Table II have been rearranged so that the class interval is three times as large as in the upper half. It will be seen that among animals smaller than 1.2 mm. the proportion of hermaphroditic males increases steadily while among those larger than this size the percentages decrease from 77 to 50. On the other hand, there is only a small proportion of hermaphroditic females among the smaller animals, but among the larger ones the percentage increases from 23 to 50. Approximately speaking, before the animals reach 1 mm. in diameter the proportion of males increases very rapidly, indicating an early proliferation of the male reproductive cells giving rise to the types of males whose gonads are predominantly masculine in appearance. After the animals grow to a size greater than 1.5 mm. in diameter the female reproductive cells overtake the male in development, thus bringing about a decrease in the proportion of hermaphroditic males after the 1.8 mm. size group and an increase in the predominantly feminine type. The decrease in the proportion of animals which are predominantly masculine continues until the animals are about 2 mm. in diameter, from which stage onward the two sexual types are equally numerous (Fig. 3).

The Relation between Functional Types and Size

In addition to the above morphological types, we can analyse our material into functional types. From the structure and the place of occurrence in the body of mature germ cells each *Limacina's* state of reproductive activity at the time of fixation can be deduced. Thus, when mature spermatozoa are seen in the gonoduct, or at the base of the penis, the individual is obviously functioning as a male and can be classified as an *active male*. When mature ova are found in the ovotestis the indi-

vidual can be considered as a *mature female,* while the presence of ripe eggs in the gonoduct indicates that the individual is a *spawning female.* The simultaneous presence of mature spermatozoa in the gonoduct and ripe ova in or out of the gonad is an indication that the individual is a *functional hermaphrodite,* to use Coe's terminology. The occurrence of these functional types is shown in Table III.

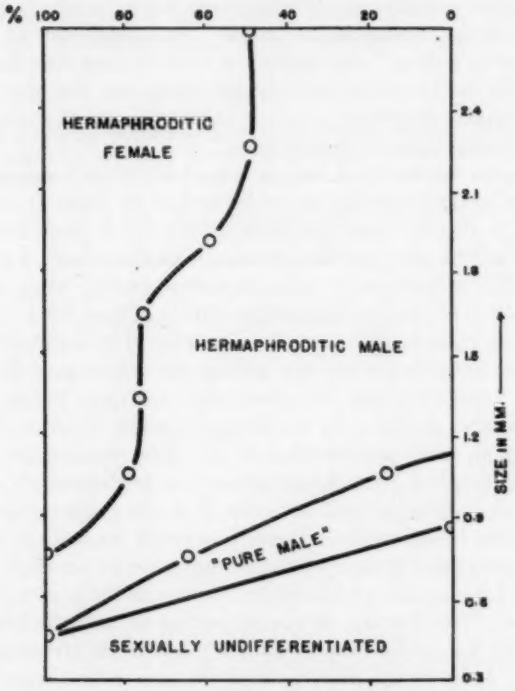

FIG. 3. Diagram showing the relation between size and various morphological types of gonads of *Limacina retroversa* (expressed in percentages of the total of each size group).

It has been observed that some individuals as small as 0.7 mm. in diameter may function as males. But the majority of specimens actively functioning as males only are between 1.2 mm. and 1.8 mm. in diameter (column 4). In larger-sized groups their place is taken by the females and individuals functioning both as male and female simultaneously.

The proportion of mature females with ripe eggs in the gonad, on the contrary, increases with the increase of size. The class with the

largest number of mature females, as shown in Table III, is the 2.1 to 2.4 mm. group. The smallest *Limacina* with mature ova is more than 1 mm. in diameter. At the time of fixation many mature females were in the act of spawning, as evidenced by the presence of ripe ova at various points along the gonoduct. Roughly speaking, about half of the mature females in each size group were in the act of spawning when fixed.

The proportion of the third functional type, functional hermaphrodites, in each size group is shown in column 6 of Table III. The chief occurrence of this type of animal is among the larger-sized specimens. It will be seen from Table III that although both specimens of the 2.4–2.7 mm. size group are functional hermaphrodites, the number of individuals used is so small that no great importance can be attached to the

TABLE III

Proportion of different functional types of *Limacina retroversa* expressed as percentage of each size group irrespective of the time of collection. Class interval: 0.3 mm.

1	2	3	4	5	6	7
Size groups (mm.)	Total number	Undifferentiated	Active male	Mature female	Functional hermaphrodite	Without signs of activity
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
0.3–0.6.....	6	100	0	0	0	
0.6–0.9.....	52	30	17	0	0	53
0.9–1.2.....	41	0	39	15	5	41
1.2–1.5.....	44	0	48	9	14	29
1.5–1.8.....	43	0	47	18	21	14
1.8–2.1.....	19	0	5	42	10	43
2.1–2.4.....	4	0	0	50	50	0
2.4–2.7.....	2	0	0	0	100	0

deduced percentage of this type of animal. Until more large specimens are studied it seems better to leave this group out in our examination of the relation between the functional types and size. In the 2.1–2.4 mm. size group mature females and functional hermaphrodites are equally numerous.

When the gonads of these functional hermaphrodites are examined and classified in terms of the proportion of masculine and feminine tissue in them the following relationship is seen.

Type of gonad:	Percentage of specimens
With more than 75% male tissue	57%
With 50%–75% male tissue	33%
With 25%–50% male tissue	10%
With less than 25% male tissue	0%

Most functional hermaphrodites have more than 75 per cent of the gonadal tissue made of male reproductive cells, one-third of them have a 50 per cent to 75 per cent male type of gonad, while no *Limacina* with less than 25 per cent male tissue in the gonad, that is, no very pronounced female type of individual, functions as hermaphrodite. Since the gonad of this species of pteropod changes from predominantly male to predominantly female type as the animals grow (see Fig. 3), this relation between the proportion of functional hermaphrodites and the amount of masculine tissue in the gonad can be interpreted to mean that animals possessing "more than 75 per cent male" type of gonad later have their female germ cells developed while the male phase of reproduction is still functioning, thus giving rise to a large proportion of hermaphrodites functioning as male and female at the same time. By the time the gonads change over to the predominantly feminine condition the male phase of sexual activity also disappears and hence few or no functional hermaphrodites are seen among individuals with a pronounced feminine type of gonad.

Figure 3 summarizes the facts about the relation of size to sexuality. Sexual differentiation commences during the time when the animals are between 0.6 and 0.9 mm. in diameter. The male germ cells proliferate in smaller-sized individuals giving rise to the "pure males" and hermaphroditic males before the animal reaches 1.2 mm. in diameter. The proportion of "pure males" decreases with increase in size. As the size increases further, the proportion of hermaphroditic males decreases, while that of the hermaphroditic females becomes greater and greater until the two sexual types are equal in number among animals greater than 2 mm. in diameter.

Functionally the situation is summarized in Fig. 4. The male phase starts among smaller *Limacina* than the functional female and hermaphroditic phases. The highest proportion of actively functioning males is found among animals 1.2 to 1.8 mm. in diameter. The large proportion of mature females and functional hermaphrodites occurs among larger animals replacing the male phase in those size groups. Females become mature after they have attained a diameter greater than 1 mm. It is probable that mature females and functional hermaphrodites are equally numerous among animals greater than 2 mm. in diameter. The percentage of each of these three functional types among the different size groups is shown in Fig. 4.

Seasonal Changes in the Reproductive Cycle

When the condition of the reproductive system of individuals collected at different times of the year is examined comparatively it is found

that, as a general rule, in winter individuals are small protandric males and that later hermaphrodites and functional males and females appear. We shall first take up the morphological types of sexual individuals and the question of the proportion of these sexual types in different months, then the appearance of different sexual activities at various times of the year and lastly the question of the reversal of sex predominance. It has

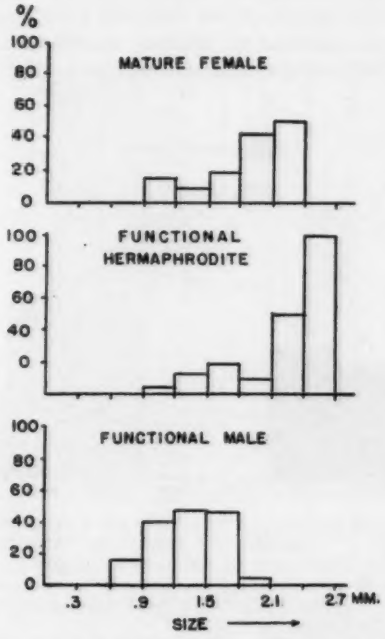

FIG. 4. Distribution of the three functional types of *Limacina retroversa* among various size groups. Functional males occur among smaller-sized animals than functional hermaphrodites and mature females.

been shown by Redfield (1939) that "population A" can be clearly differentiated from "population B" from December up to May and after that time there is a good deal of mixing of populations with "population B" predominating over the descendants and remnants of "population A" and new recruits into the Gulf. In the following description data from December to May refer to "population A" and those after May, to the mixed populations.

Change in Gonad Structure

Young sexually undifferentiated individuals were found among the specimens taken in each month except March and April. Figure 5 shows the percentage of the individuals taken each month which are sexually indefinite. During the winter months of December and January "population A," which entered the Gulf then, contained 14–16 per cent of sexually undifferentiated animals. In March and April these animals drifted to the western portion of the Gulf and grew larger, as shown by Redfield, and there remained no sexually undifferentiated individuals. After May, a portion of the animals examined, 19–20 per cent, were

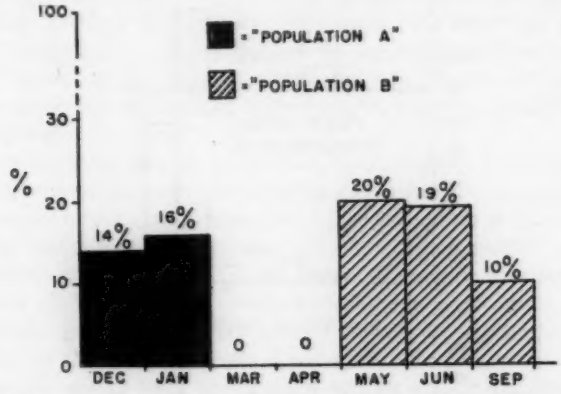

FIG. 5. Histogram showing the percentage of sexually undifferentiated individuals in each month. "Population A" solid black, "population B "shaded with straight lines. About 15 per cent of the material caught (in the winter) from "population A" and 20 per cent from "population B" are sexually undifferentiated. Absence of immature material in March and April indicates that members of "population A" are all differentiated by March.

again sexually indefinite. This coincides with the entrance into the Gulf of a new population in the east and the spawning of "population A" in the west portion of the basin. As far as "population A" is concerned, sexual differentiation is completed by the month of March and all the individuals of this population were either developing in the direction of males or hermaphrodites.

Animals which were sexually differentiated have been divided into male or female type according to whether they had more or less than 50 per cent of their gonadal tissue made of male reproductive cells. The proportions of these male and female types among sexually differentiated *Limacina* collected each month are shown in Fig. 6. In this figure the

material before May refers to "population A" and that after May to the mixed populations, with "population B" as the predominating constituent. It will be seen that in December and January nearly all the sexually differentiated *Limacina* were predominantly male. After these months the proportion of predominantly male individuals became reduced: in April they constituted only 40 per cent, while in May the two sexual types were equally numerous. After May the proportion of predominantly male animals became more numerous again among the mixed populations.

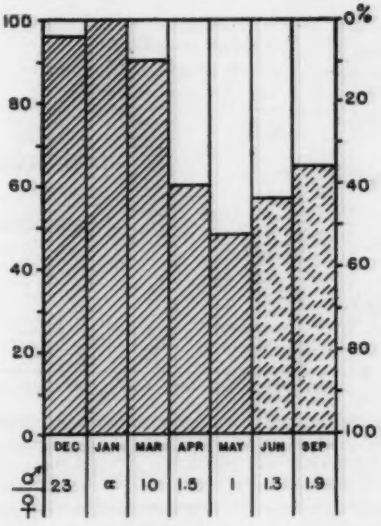

FIG. 6. Proportions of hermaphroditic males and hermaphroditic females among sexually differentiated *Limacina retroversa* in each month. Unshaded area represents hermaphroditic females, shaded areas hermaphroditic males. Area shaded with continuous lines refers to "population A," that with broken line, to "population B." ♂/♀ refers to the ratio of sex predominance in each month.

When the change in the percentage of the two sexual types among sexually differentiated *Limacina* from month to month as shown in Fig. 6 is calculated into ratios of sex predominance (the number of predominantly male individuals over those predominantly feminine) a change from a high value to unity is seen. These values are appended at the bottom of Fig. 6. It will be seen that in December and January this value is very high, but in May it is equal to one, while among the mixed populations the value is again in favor of the male type in the later part of the year.

In Fig. 7 are shown the proportions of "pure males," animals with little or no feminine tissue in the gonad, and "less than 25 per cent" male (very pronounced feminine) types among the sexually differentiated individuals in each month. About 45–55 per cent of sexually differentiated *Limacina* in December and January were "pure males," the rest being hermaphroditic males. In March the proportion of "pure males" was reduced greatly and from April onward this type of individual was not seen any more. On the other hand, pronounced feminine individuals with less than 25 per cent of the gonadal tissue made of male germ cells were not seen before May. It will also be noticed that

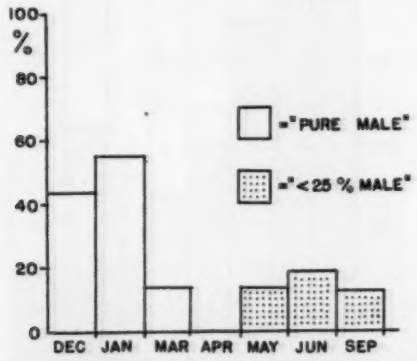

FIG. 7. Monthly appearance of "pure males" and hermaphroditic females with less than 25 per cent of their gonadal tissue consisting of male germ cells. Histograms before April refer to "population A" and those after April to "population B" with mixed populations. "Pure males" represented by unshaded areas; "less than 25 per cent male" type of hermaphroditic females represented by shaded areas.

the occurrence of this type of extreme female in the later part of the year has been always low in comparison to the other types.

Change in Reproductive Activity

The reproductive activities of *Limacina retroversa* show a parallelism with the morphological changes associated with the advance of time. During December and January when "pure" and hermaphroditic males constituted nearly all the groups of sexually differentiated individuals no hermaphroditic females with mature ova in the gonad were seen in the specimens examined, while spermatozoa, on the other hand, were evidenced as being liberated by the fact that mature male germ cells were

found in different parts of the gonoduct. It is in March, however, that individuals with mature ova in their ovotestes began to appear. This type of individual continued to increase in proportion until May. In May animals had been collected and fixed during their spawning activity. Later in the year all the different types of functional individual were seen. The occurrence of these types of functional individual in the various months is shown in Table IV.

TABLE IV

Distribution of functional types of *Limacina retroversa*, expressed in percentages, in different months.

1	2	3	4	5	6
Month	Number studied	Active males	Mature females	Spawning females	Functional hermaphrodites
		per cent	*per cent*	*per cent*	*per cent*
Dec.	24	63	0	0	0
Jan.	26	58	0	0	0
Mar.	22	55	5	0	0
Apr.	25	32	20	0	8
May	23	17	43	30	26
June	21	19	38	19	19
Sept.	26	19	27	12	35

It will be seen from column 3 of this table that individuals ready to liberate spermatozoa are seen throughout the year, but that the proportion is higher in the earlier part of the year than in the later and that the reduction of the number of active males is accompanied by an increase in the number of individuals functioning as females (columns 4 and 5) and those acting as hermaphrodites (column 6) after April. A comparison of columns 4 and 5 will show that spawning is found after the mature females were observed in March. It has been found in the section on the relation between size and sexuality that about half of all the mature females examined were fixed during the act of spawning. From column 5 of Table IV it is seen that in May about three-quarters of the mature females were fixed in this stage, while in June the proportion of spawning females amounted to half of the matured ones, and in September, less than half. This indicates a higher spawning activity in May than in the other months. The fact that no spawning individuals were seen among the mature females in April does not exclude the possibility that egg-spawning took place in this month but that the individuals did not happen to be fixed with eggs in the gonoduct and selected for examination in this study. We may consider spawning to start be-

tween April and May and continue to the fall with May as the most important period for breeding.

From the above examination of the reproductive system of *Limacina* in terms of time it appears that among "population A," which entered the Gulf of Maine first, all the individuals were sexually differentiated by the beginning of March. In winter, a great majority, if not all, of them were "pure males." The number of such "pure males" decreased in March and they were not seen in April, giving place to individuals with a lesser degree of masculinity, to functional hermaphrodites and eventually to hermaphroditic females with very little masculine tissue in their gonads. The proportion of male tissue in comparison with the female in the gonad decreased as the animals grew. By April 40 per cent of the specimens collected had the feminine tissue so well developed as to warrant their being classified as hermaphroditic females. Animals with "less than 25 per cent male" gonads occurred after the "pure males" had disappeared from the collections. In May the two sexual types were equal in number. After May the bulk of the species in the Gulf was derived from the recently arrived "population B" which mixed with the descendants and remnants of "population A" and other new recruits. Among the mixed population sexually undifferentiated individuals were seen again and all the structural types were present.

The functional phases of reproduction are summarized in Fig. 8. More than half of the protandric males started to function in winter and the male phase continued into spring and summer as it became gradually replaced by the female and functional hermaphroditic phases of reproduction. Females began to attain maturation in March and the proportion of mature females increased rapidly in April and May. Spawning of ova commenced between April and May, being highest in the latter month. The largest proportion of functional individuals, including the male, female, and hermaphroditic types, is seen in May when half of them were spawners of eggs and the other half were males and hermaphrodites in function. These individuals were seen in the months from June to September also. It appears that May is the most important period for spawning though the reproductive activity was carried on into the autumn.

Change in Sex Predominance

A change in the value of the ratio of the sexes during growth is generally used as one of the indications of a change of sex. As the term "sex ratio" is not very appropriate for monoecious forms where both male and female reproductive tissues are present in the gonad,

it seems better to speak of sex predominance and ratio between male and female predominance. The decrease in the value of this ratio from small to large-sized animals may be due, in general, to any one of the following causes: (*a*) Sexual dimorphism such that the males are habitually smaller than the females, (*b*) differential mortality, that is, more males than females die as the animals grow, and (*c*) sex reversal or an actual change in the condition of the reproductive system as the animals grow. There is no evidence from our material either to show that there

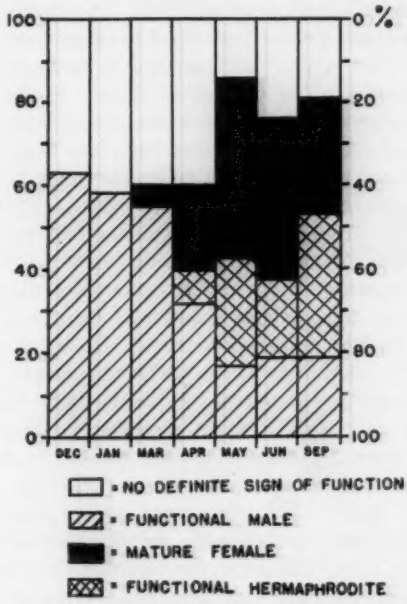

FIG. 8. Monthly distribution of the three functional types of *Limacina retroversa*.

is a sexual dimorphism or to indicate a differential mortality which may be interpreted as the cause of the appearance of a higher proportion of males among smaller-sized *Limacina* and a greater number of females among larger ones. The third alternative, that a change in the predominant sexuality takes place as the animals grow, however, has much evidence in its favor. In the first place, it has been shown above that there is a parallelism between the changes in the ratio of sex predominance with reference to size and that with reference to the advance of the season: male types dominate among smaller-sized *Limacina* and dur-

ing the winter months, and this excess of males decreases as the animals become larger and also as the season advances, and eventually the two types become equal among still larger-sized specimens and at the height of the breeding season in May. This parallelism indicates a change in the sexuality, both in structure and function, of the individuals as they grow.

In the second place, it has already been shown in a previous paper that there is no clear line of demarcation between male and female among *Limacina,* but that hermaphroditic male and female merge into each other and that a continuous series can be made out. As the size of the animals increases, a greater and greater portion of the gonad appears to contain female tissue. "Pure males" are confined to small-sized animals and in the winter months. The continuous change in the proportion of male and female reproductive tissues in the gonad culminating in many functional hermaphrodites and females with less than 25 per cent of the tissue of their sex glands made of masculine elements argues in favor of a reversal of sex predominance in this species. Furthermore, there is no "pure female" among *Limacina:* all the hermaphroditic females are large and apparently derived from male types.

Thirdly, a comparison of the size distribution of the different sexual types in the various months also shows that there is a change in sex predominance. In Fig. 9 the proportions of (*a*) sexually undifferentiated young, (*b*) individuals which are predominantly masculine and (*c*) those which are predominantly feminine, have been indicated in the histograms prepared by Redfield showing the distribution of size among all the specimens taken during each cruise. It shows the earlier occurrence of protandric males and later appearance, in March and April, of female types. The appearance of the female type among larger size groups suggests that they are transformed from the protandric individuals rather than from the very small undifferentiated ones.

From these morphological male and female types of *Limacina* functional hermaphrodites have also been indicated in the same figure (Fig. 9). These functional hermaphrodites increase from a very small proportion in April to a maximum in May and then decline in June. This change in the relative proportion of this type of individual supports the idea mentioned above that protandric males develop ovarian tissue while they are still functioning as males and eventually become functional females.

Finally, if the modal class of each month's collection is taken as representative of the total catch, the phenomenon of change of sex predominance can be brought out when the different modal size groups are compared. Analysis of the size frequency distribution showed that

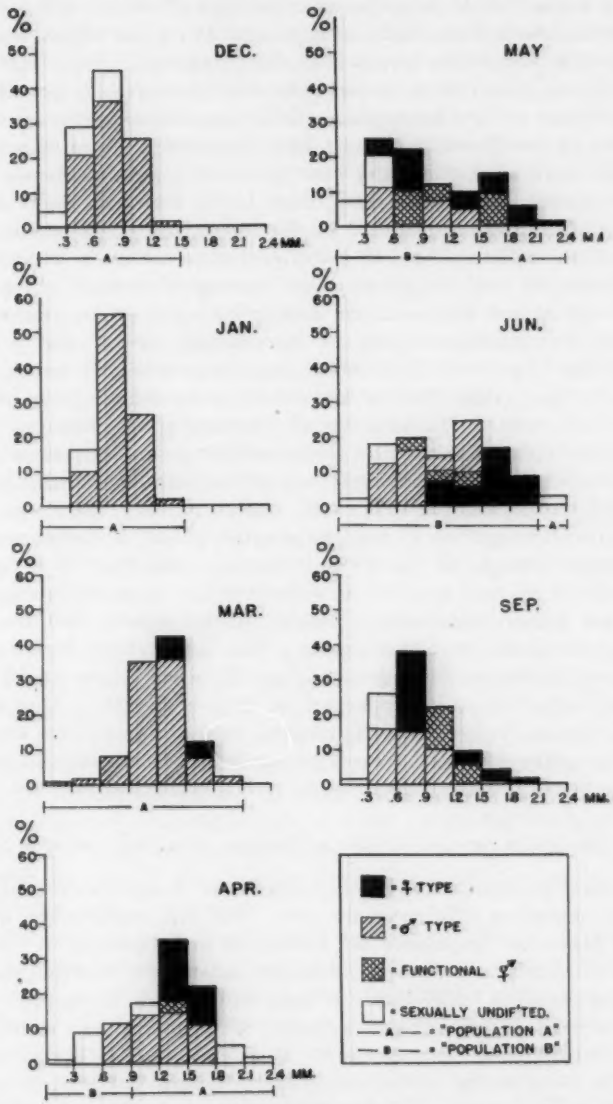

FIG. 9. Distribution of different types of *Limacina retroversa* among various size groups in each month. The proportion of each sexual type is added to the histogram showing size distribution prepared by Redfield (1939).

there is a single mode from December to April (Redfield, 1939). This modal class shifts from smaller to larger size during this period, showing a general growth of the members of the population. The sexual condition of the gonads of the modal class shows that not only growth, but sexual change has also taken place. In the modal class of the December material all the differentiated individuals are males and over 60 per cent of them have more than three-quarters of their gonadal tissues composed of male reproductive cells. Half of the males are ready to liberate spermatozoa. In January all the individuals of the modal class are predominantly male and 65 per cent of them are ready to discharge the product of their sex glands, thus showing an increase in the proportion of sexual differentiation with growth and an increase in the number of individuals capable of inseminating others. But it is in March that 11 per cent of the modal class are seen to have more female tissue in their gonads; the rest are still all predominantly male, half of which are ready to discharge or are discharging spermatozoa. The proportion of females increases to one-half of the modal class in April and some of the individuals begin to function both as male and female simultaneously. Of the two modal classes in May, the larger one shows, in the condition of the reproductive system, a continuation of the general changes of the previous months. For there is a further reduction of the male type and an increase of the functional hermaphrodites and actively functioning females. In this month, when the egg-laying individuals are added together, that is, spawning females plus functional hermaphrodites, they make up the whole of the modal class (see the right half of the histogram for May in Fig. 9). Thus if the modal class found by Redfield is taken as representative of the material collected, observations on the sexual condition of these groups give a consistent story of the change of sex predominance and function.

Relation between Sexual Phases and Distribution

When the sexual condition of populations A and B are compared several interesting differences are seen. The first characteristic of the sexual phases of "population B" in contrast to "population A" is that sexual differentiation seems to commence earlier with reference to size. In "population A" individuals as large as 0.85 mm. in diameter have been seen as undifferentiated sexually, while the largest undifferentiated individual of "population B" is only 0.6 mm. in diameter. In addition to the earlier differentiation of the gonads, a second difference is seen in the manner of differentiation between the two populations. Instead of the male elements in the gonads always taking the lead in

development so as to bring about protandry, some individuals among "population B" have both types of sexual cells developed at nearly the same time in addition to protandry, so that proterogyny and hermaphroditism appear. In view of this type of development, small-sized mature hermaphroditic females and functional hermaphrodites may also be interpreted as derived directly from young undifferentiated individuals with the protandric stage omitted. In "population A" such a situation is not seen. The third point of difference is that the development of the female elements of the gonads of individuals in "population B" is also earlier. This can be seen when the minima of the size of the individuals which achieved maturation of ova in the two populations are compared. The minimal size of *Limacina* with mature ova is 0.8 mm. in diameter in April and 1 mm. in May among members of "population B," while among "population A" the minimal size of mature females is 1.3 mm. in April and 1.67 mm. in May. It is possible that these small mature females in "population B" did not pass through a protandric stage of noticeable length, though other specimens of the same population have a distinct young male phase preceding the female. In "population A" the smallest functional hermaphrodite is 1.5 mm. in diameter in April and 1.6 mm. in May, while during the same months functional hermaphrodites as small as 1.1 mm. in diameter are found among "population B."

In Fig. 9 the right portion of the May histogram which has been attributed to "population A" by Redfield is very similar to that of April in the distribution of sexual types. In both cases hermaphroditic females occupy the large size groups with functional hermaphrodites between them and the small-sized, predominantly male individuals. On the other hand, the left portion which represents "population B," has a size distribution comparable to those of the winter polygons but the sexual phases are quite different from them. Here the hermaphroditic females and functional hermaphrodites appear among protandric males in small size groups, which in the previous months only contain protandric and "pure" males.

All these differences in the sexual condition of the two populations seem to point convergingly to one underlying factor, namely, that during the warmer part of the year sexual differentiation takes place sooner than in the colder time and the sexual phases may be telescoped together when differentiation is speeded up in the summer. This is in line with the previous observations made on *Ostrea* by authors on both sides of the Atlantic (Coe, 1932, 1936; Needler, 1932; Orton, 1926; Spärck, 1925). However, this does not exclude the possibility that there is a

difference in the origin of "populations A and B" which may account for some of the observed differences in sexual development.

Comparison of the Reproduction of Limacina retroversa with Other Mollusks

As the reproductive history of no other Opisthobranchiata has been described, it is not possible to compare the reproduction of *Limacina retroversa* with that of the members of the same genus or family or even order. Only in a general way can the sexual sequence of this form be compared with that found among other mollusks.

In the winter months *Limacina* which entered the Gulf of Maine are of small size and sexually undifferentiated. As they grow this initial phase is followed by protandry in which nearly all the individuals are predominantly male. The protandric phase is succeeded by a hermaphroditic phase during which the animals transform from a more masculine to a more feminine condition. In the final phase the individuals are predominantly female in structure and generally function as hermaphrodites, liberating both ova and spermatozoa, or as females only. This type of sexual transformation conforms in a general way with that found among gastropods. The sequence approximates most closely to that of the prosobranchiate gastropods such as *Valvata tricarinata* worked out by Furrow (1935) and *Patella vulgata* by Orton (1928). But the time required for the transformation of sex is different. Orton observed that the male *Patella* transformed into female at the age of one year, while Furrow found that in *Valvata* the different sexually functional phases followed each other very closely and the protandric phase was very short. However, in *Limacina retroversa* the change in sex predominance seems to take place during a period of two or three months. For instance, in one population which entered the Gulf of Maine in December and January, the protandric males were transformed into females from March to May. In the warmer part of the year this transformation required a shorter time for its completion.

Summary

1. In the winter months sexual differentiation commences when the animals are between 0.6 and 0.85 mm. in diameter. In the summer sexual differentiation starts when the animals are smaller.

2. The early proliferation of the male reproductive cells leads to a protandric phase. The "pure males" give place to hermaphroditic males and later to hermaphroditic females as the size of the animals becomes greater than 1 mm. in diameter. After the specimens are 1.5

mm. in diameter the female reproductive cells overtake the male in development, bringing about a decrease in the proportion of hermaphroditic males and an increase of the opposite sexual type. This change continues until the two types are equal, when the animals are 2 mm. or more in diameter.

3. Functionally the male phase starts among smaller *Limacina* than do the functional female and hermaphroditic phases. The highest proportion of actively functioning males is found among animals 1.2 to 1.8 mm. in diameter. Among larger animals the functional hermaphroditic and functional female phases replace the male phase. Hermaphroditic females begin the functional phase after they are greater than 1 mm. in diameter.

4. Members of a population which entered the Gulf of Maine in the winter were all sexually differentiated by the beginning of March. Nearly all of these individuals developed into protandric males, about half of which were "pure males." The number of such "pure males" decreased in March and disappeared in April, being replaced by animals with greater predominance of feminine tissue in the gonads. By April about 40 per cent of the specimens were hermaphroditic females and in May the two sexual types were equal.

5. The male functional phase began in winter and continued into spring and summer. Hermaphroditic females matured in March and their proportion increased in April and May. Spawning of ova commenced by the end of April. The largest proportion of functional individuals of all types was seen in May.

6. Reasons are advanced for the belief that the change in sexual constitution of the populations is due to a reversal of sex predominance of the individuals.

7. The sequence of the sexual phases of *Limacina retroversa* agrees in a general way with those found for other members of the gastropod group of mollusks, particularly that of *Valvata*.

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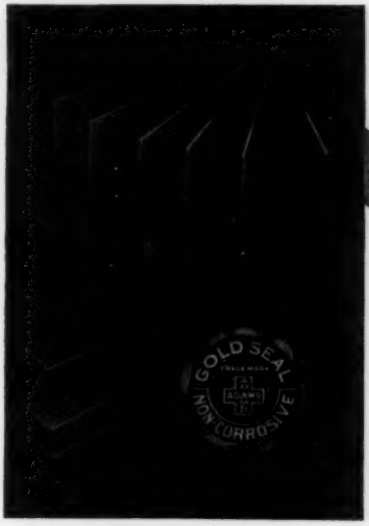

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